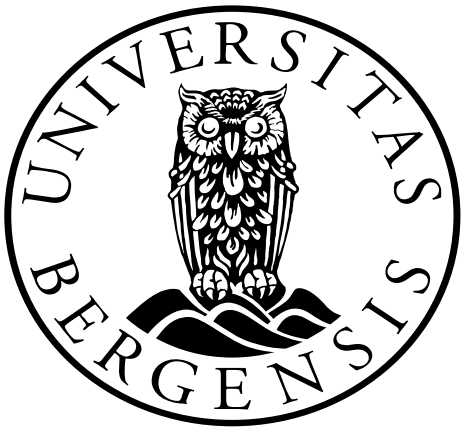
Modelling night shift work and gastrointestinal function.

Maiken Birkelid



MAPSYK360, masterprogramme in psychology,

behavioral neuroscience.

UNIVERSITY OF BERGEN

FACULTY OF PSYCHOLOGY

AUTUMN 2019

Supervisor: Jelena Mrdalj

Department of Biological and Medical Psychology, University of Bergen, Norway

Abstract

Shift work is associated with negative health outcomes related to the gastrointestinal system such as risk for developing gastrointestinal diseases, irritable bowel syndrome and colorectal cancer. Previous research suggests circadian rhythm disruption is an underlying mechanism. In my master thesis, I investigate the consequences of shift work in a rat model on protein markers in feces and gene expression in intestinal mucosa, and discuss possible mechanisms for the observed changes.

To mimic human shift work, rats were kept awake by being placed in rotating wheels during their "work shifts" for 8 h. One group worked in their rest phase (RW) another during their active phase (AW). Another two groups not exposed to the "work shifts" served as time-matched controls as they were sacrificed at same time points as RW and AW. The simulated shift work protocol lasted for three consecutive days. A time-of-day variation in oxidative stress marker was found in protein markers in feces, but these markers were not affected by shift work. In addition, a time-of-day variance was observed in all, with the exception of one, measured clock genes in colonic intestinal mucosa. The clock gene expression were significantly altered in RW. Expression of a clock-controlled gene involved in cell proliferation was also altered in RW, and not in AW, indicating a possible mechanism behind the association of increased colorectal cancer seen among shift workers. In conclusion, three days of rest phase work induced changes in clock gene expression in colonic mucosa tissue.

Key words: Circadian rhythm, gastrointestinal tract, night shift work, colon, shift work

Sammendrag

Skiftarbeid er assosiert med negative helse konsekvenser relatert til mage-tarm kanalen som økt risiko for å utvikle mage-tarm sykdommer, irritabel tarms syndrom og kreft i tykktarm eller endetarm (kolorektal kreft). Tidligere studier foreslår forstyrrelse i døgnrytme som en mulig underliggende mekanisme. I min master oppgave benytter jeg en rottemodell for å undersøke konsekvensene av skiftarbeid på protein markører i avføring, og på gen-uttrykk i slimhinnelaget (mucosa) fra tarmveggen. Deretter diskuterer jeg mulige mekanismer for de observerte forandringene.

For å etterligne menneskelig skiftarbeid ble rotter plassert i roterende hjul for å holdes våkne under «skiftarbeidet» som varte i 8 timer. En gruppe med rotter jobbet i hvile-fasen (RW), mens en annen jobbet i den aktive-fasen (AW). I tillegg var det to kontroll grupper som ikke ble eksponert for «skiftarbeid» som ble avlivet på samme tidspunkt som RW og AW slik at de kunne sammenlignes. Simulert skiftarbeid varte i tre sammenhengende dager. Variasjon i tid på døgnet ble observert for oksidativt stress protein markør i avføring, som var upåvirket av skiftarbeid. I tillegg ble det observert en tid-på-døgnet variasjon i alle målte klokkegener, med unntak av én, i tarmslimhinnen i kolon. Uttrykk av klokkegener var signifikant endret hos RW. Uttrykk av et klokke-kontrollert gen som er involvert i celledeling var kun endret hos RW. Dette er en indikasjon på en mulig mekanisme bak assosiasjonen av økt kolorektal kreft hos skiftarbeidere. Konklusjonen er at tre dager med arbeid i hvilefasen er nok til å forårsake forandringer i uttrykk av klokkegener i slimhinnen i kolon.

Nøkkelord: Døgnrytme, mage-tarm kanalen, nattarbeid, kolon, skiftarbeid.

Acknowledgement

First of all, I would like to thank my supervisor Jelena Mrdalj for your guidance, valuable feedback and support throughout this period. Thank you for always being available, having time for my questions and share your passion and knowledge for science. You have challenge me on multiple levels, by encouraged me to step out of the comfort zone and by having faith in me has made me grow as a person. I have learned a lot from you.

I would also like to thank Janne Grønli, especially for assisting in the application of "Meltzer høyskolefond" project stipend, and for valuable input and suggestions throughout the design and experiment period.

I would also like to thank all the people who have assisted me during the experiment, data collection or in the analysis process in the laboratory. Ingebord Brønstad for taking the time to train and guide me during all my protein analysis. Silje Skrede for offering your expertise in RT-PCR. Anne Marita Milde for assisting in collection of tissue in the lab and share your knowledge. Bodil Bjørndal and Kari Merete Ersland for technique demonstration of how to remove tissue and RNA isolation, and Nina Harkestad for all your assistance in the laboratory and always being available to answer and discuss my questions.

My contribution to the dataset in this thesis

In the second semester of my master programme, August 2018, I was invited to take part in the development of this project by led Jelena Mrdalj. The project was to be performed in collaboration with National Center of Functional Gastrointestinal Disorders led by Birgitte Berentsen, which is experienced in clinical and experimental gastrointestinal research. In the beginning the main aim of the project was to look at protein markers in feces following simulated night shift work in rats (the cost for these analyses were funded by National Center of Functional Gastrointestinal Disorders). However, we wanted to expand the project, and with the helpful guidance from Janne Grønli, leader of Bergen Stress and Sleep group, I applied for funding through "Meltzer Høyskolefond". In March 2019 I was awarded a total of 75 000 NOK, which made it possible to significantly expand the project to also include gene expression analyses, and tissue collection from a new group of animals serving as controls.

I have contributed to the design of the experiment, technical set-up of the experimental equipment, surgical implantation of telemetric transmitters (for recording of sleep and circadian rhythm of body temperature and activity; these data were used in another study), post-surgery care, collection of data (tissue and feces), and analyses of data presented in this thesis. The collection of data was performed throughout the period of December-Januar 2018/19 and was done in close collaboration with the research team. This included daily care of animals; checking health status and providing food and water. Animals were weighted before and after "work shifts", placed in automatically rotating wheels, data from telemetric recording was collected both in home cage and in the rotating wheels in a separate experimental room. This was performed both in the daytime and during the night, with only red light as light source. At the end of each shift the rotating wheel was cleaned, feces sampled, and animals returned to their home cage.

At the end of experiment, I organized, prepared and assisted in euthanizing animals for dissection. Tissue was collected from several organs and I had the responsibility of collecting feces and intestinal samples. I was trained beforehand by Bodil Bjørndal, Department of Clinical Science, in a technique where mucosa is separated from the intestine wall. I also did most of the molecular analyses myself; I did all protein marker analyses with the use of the ELISA method for which I was trained and supervised by Ingeborg Brønstad, Department of Clinical Science. For gene expression I performed the initial step of isolating mRNA from tissue and preparing the samples for real-time polymerase chain reaction (RT-PCR). I was trained beforehand by Kari Merete Ersland. The last step of gene expression analysis by the use of RT-PCR was performed by Silje Skrede at Department of Clinical Science. With help from Jelena Mrdalj I did statistical analyses on the molecular results. I am very grateful for being invited to take part in this project at the beginning and during the development throughout the whole period. Moreover, I am also very lucky to have been able to take an active part in each step from the design of the experiment, execution of the experiment, and that I received the opportunity to be trained in the laboratory to do the molecular analysis first hand, which all were important phases that led to the completion of this master thesis.

Table of contents

List of figures

1.1 Night shift work

The number of people working in shifts is generally increasing around the world because of developing world economy and worldwide communication that demand around the clock availability. This has resulted in a percentage of 20 to 25 % of the work force working shifts in industrialized countries (Costa, 2010). Shift work is not clearly defined and definition varies between countries and industries with regards to duration, timing and rotation (schedule). This is mostly because each country has different legal rules and social obligations. However, shift work is generally defined as work that takes place on a schedule outside the "traditional" 9am – 5pm on workdays, and is often thought of as synonymous with flexible, irregular, variable and non-standard working hours (Wickwire, Geiger-Brown, Scharf, & Drake, 2017).

A night shift is a form of shift work which is defined by the Norwegian Employment Protection act (§10.11) as work taking place between 9pm and 6am. Statistics from the 6th European survey on working condition showed that about 19% of all employees work a night shift at least once a month (European Foundation for the Improvement of Living and Working Conditions, 2015). Generally, night employment leads to a shortening of sleep time and a reduction of quality of sleep (Costa, 2003; Torsvall, Akerstedt, Gillander, & Knutsson, 1989). This is because our circadian rhythm is programmed in such a way to keep us awake during the day and asleep during the night. People working a night shift have to adjust their circadian rhythm to a nocturnal regime, but it seems that irrespective of how many years shift workers have been on permanent night shift, nearly all (-97%) night shift workers do not adjust to the nocturnal regime but are still synchronized to a diurnal rhythm (Folkard, 2008).

There is a growing number of epidemiological studies that associate night shift work with increased risk for developing negative health outcomes related to metabolism and the

gastrointestinal system (Costa, 2010; Knutsson, 2003). Immediate consequences associated with night shift work in addition to sleep disturbances and fatigue, are gastrointestinal disturbances such as abdominal pain, constipation or diarrhea. Long-term night shift work is associated with a number of negative health outcomes such as gastrointestinal disease (Knutsson & Bøggild, 2010), irritable bowel syndrome (Nojkov et al., 2010) and colorectal cancer (X. Wang et al., 2015). Despite this, our understanding of causality and underlying mechanisms that explain the relationship between night shift work and gastrointestinal function is limited. The gastrointestinal tract is a complex organ harboring many different biological systems that might be susceptible to changes in the circadian rhythm.

1.2 Circadian rhythm

The term circadian is derived from the Latin words "circa" and "diem" meaning approximately a day. Circadian rhythms are endogenous oscillators with a cycle length of approximately 24 hours. They are self-sustained, meaning that they persist in a constant environment as for example constant darkness, where there are no environmental cues about the time of day. Circadian rhythms can be observed at different levels in our bodies from behavior, physiology, to cellular and molecular processes. Behaviorally the most obvious one is the sleep-wake cycle, whereas the temperature change from higher in the active phase to lower in the inactive phase can be observed at the physiological level. Further down to the molecular level the most known circadian rhythm is probably that of the stress-hormone cortisol, which peaks in the morning helping us to wake up, and with lowest values in the early evening. This rhythmicity in different functions and processes in our bodies is thought to be an evolutionary adaptive response to the earth's rotation around its axis and the resulting predictable environmental changes of light, temperature and food availability as day follows night (Vaze & Sharma, 2013).

These rhythms can be found in most organisms ranging from bacteria, plants, animals, to humans (Bell-Pedersen et al., 2005), and are thought to be adaptive in the sense that organisms can anticipate predictable rhythmic changes in the environment and coordinate temporally appropriate changes in their physiology and behavior (Yerushalmi & Green, 2009). For this to happen, these endogenous rhythms must be synchronized or entrained to the external environment. This is done by entraining-signals known as zeitgebers (German for "time-givers") (Aschoff, 1965), with light being the dominant synchronizer responsible for resetting our circadian rhythm each day, keeping it aligned with the astronomical day (Pittendrigh & Minis, 1964).

1.2.1 Circadian rhythm regulation. Circadian clocks are found in virtually all cells in the body (Reppert & Weaver, 2002), but in order for them to act in synchrony there must be one master biological clock, or a pacemaker that synchronizes downstream peripheral clocks in a hierarchical fashion. One area that sits at the base of the hypothalamus, just above the optic chiasm fits this description and is called the suprachiasmatic nuclei (SCN). Evidence for this area as a master biological clock came first from early lesion experiments in rats, which showed that ablation of SCN eliminated rhythmicity in behavior (Moore & Eichler, 1972; Stephan & Zucker, 1972), and second, when removed cells from the SCN were cultivated in vitro and continued to show their own rhythmicity (Welsh, Logothetis, Meister, & Reppert, 1995). The third and final evidence for this clock working as an autonomous pacemaker was demonstrated in a series of experiments where hamsters got transplanted SCN from donors with different circadian periods, which always resulted in expression of the genotype of the donor and not the host (Ralph, Foster, Davis, & Menaker, 1990). No other tissue, either in the brain or periphery has shown the same characteristics.

The fact that circadian rhythms share some of the same features across different species gave clues that the underlying molecular mechanisms involved in generating rhythmicity might be the same, but knowledge about the mechanism of how the circadian clock operates was not unfolded until clock genes were discovered, first in the fruit fly and later in mammals (Kolker & Turek, 1999; Panda, Hogenesch, & Kay, 2002). The clock mechanism in SCN and peripheral organs consists of autoregulatory transcriptional-translational feedback loops involving multiple clock genes (Reppert & Weaver, 2002).

The primary feedback loop consists of the clock genes *Bmal1* (Brain and Muscle ARNT-like 1) and *Clock* (Circadian Locomotor Output Cycles Kaput) which activate transcription of other clock genes known as period genes (*per1, per2, per3*) and cryptochrome genes (*cry1* and *cry2*), by binding to what is known as E-box and E-box-like enhancer sequences in their promoters. Transcriptional activation results in messenger-RNA (mRNA) exit the nucleus and being translated by ribosomes in the cytoplasm, where larger protein molecules are formed based on the transcripts. These protein molecules are susceptible to degradation unless they form dimers (a pair of molecules). PER/PER, PER/CRY, CRY/PER dimers will then translocate to the nucleus and interact with *Bmal1* or *Clock* to block activation, thus functioning as a negative feedback loop and regulating transcription of their own genes as well. Over time the PER/CRY dimers will degrade and the process will start over (Ko & Takahashi, 2006).

A secondary feedback loop in the circadian cycle is regulated by the retinoic acid-related nuclear receptors called *ROR*α (retinoid-related orphan receptor, alpha) and *RevErb-*α (nuclear receptor subfamily 1, group D, member 1) which regulate the cyclic expression of *Bmal1* (Solt, Kojetin, & Burris, 2011). In short, *ROR*α activates and *RevErb-*α inhibits transcription of *Bmal1* during the circadian night (Haus &

Smolensky, 2013). These nuclear receptors regulate gene transcription in response to environmental stimuli and also play an important role in cell proliferation and metabolism in peripheral organs. Taken together, these two interlocking feedback loops are believed to be the driving element that generate the regulation of approximately 24 h period of molecular oscillation within individual cells throughout organisms (Ko & Takahashi, 2006). Moreover, clock genes can mediate transcription of other genes containing an E-box enhancer element; by doing this they can enhance transcription of other downstream genes in a circadian manner. One example of such "clock-controlled gene" is the cell cycle checkpoint gene *Wee1* (nuclear kinase belonging to the Ser/Thr family of protein kinases) which is involved in cell proliferation, as it can delay or prevent entry of a cell to mitosis through phosphorylation (Perry & Kornbluth, 2007). The CLOCK/BMAL1 heterodimer can directly regulate the activation of transcription of *Wee1* (Matsuo et al., 2003), while PER/CRY dimer suppresses its transcription (Haus & Smolensky, 2013).

1.2.2 Circadian rhythm synchronization. This genetically determined clockwork has a period slightly longer or shorter than 24 hours, so if there are no environmental cues, this rhythm would "free run" in the sense that it would be a little delayed or advanced (depending on genetic makeup) each day, compared to the astronomical day (Czeisler et al., 1999). As mentioned initially, light is the dominant synchronizer as it is responsible to reset our biological clock in the morning. Information about light is registered by intrinsically retinal photosensitive ganglion cells (ipRGC) which are maximum sensitive to blue light between 470 to 480 nm (Berson, 2003). The information is forwarded to the SCN via the retinohypothalamic tract and other brain areas involved in regulation of sleep and wakefulness (Do & Yau, 2010). In humans,

early morning light signals a transition to the active phase forwarding waking processes, but in rodents which are nocturnal the same light information signals a transition to the rest phase thereby preparing the animal for sleep.

The activity in SCN is synchronized directly through light input, and all other tissues depend on SCN to be synchronized. SCN sends information in a hierarchal manner to peripheral organs such as the heart, liver and the digestive system so that each organ system has their own phases, which are generally delayed up to 6 h from the central clock (Kowalska & Brown, 2007). The result is a coherent time-organization of bodily processes to the upcoming daily challenges (Yamazaki et al., 2000). The exact mechanism on how this is accomplished is not completely unfolded, but it is likely through humoral, endocrine and neural pathways. It is important to mention that while light is the main synchronizer for the SCN, peripheral clocks are also regulated by other zeitgebers such as food intake, and therefore an uncoupling from the master clock in the SCN is possible. This has been demonstrated with restricted-feeding schedules where animals only have one specific time point where food is available, often in the rest phase. This causes peripheral clocks to entrain to food schedule, while the SCN clock remains entrained to the light/dark cycle (Damiola et al., 2000). Hence, the peripheral clocks come out of sync with the master clock, meaning that the aforementioned adaptive response of synchrony is broken. This misalignment of circadian rhythms of body functions may cause so-called “jet-lag” syndrome, characterized by fatigue, irritability, sleep-disturbances, digestive problems and poorer mental flexibility, which over time may cause or exacerbate severe health problems and disease (Costa, 2010).

1.3 The gastrointestinal tract

The gastrointestinal tract (GIT) is an important part of the digestive system and consists of a hollow muscular tube starting at the oral cavity, where food enters, continuing through the esophagus, stomach and intestine to the rectum and anus, where food is expelled. There are various accessory organs (salivary glands, pancreas, liver and gall bladder) that assist the GIT by secreting enzymes and other fluids to help break down food into its component nutrients. Food is propelled along the length of the GIT by peristaltic movements of the muscular walls (gut motility). In humans the GIT has an average length of 4-6 meters, and therefore has different names for locations. Although each section of the GIT has specialized functions the entire tract has similar basic structure with regional variations. This thesis will focus on the lower part of the GIT, namely the small and large intestines, therefore hereafter the GIT will refer to these areas. The GIT performs two essential roles in our bodies; 1) absorption of nutrients and 2) maintenance of immune homoeostasis (Tortora & Derrickson, 2008). This is a complex task as nutrients and other beneficial molecules need to cross the intestine wall, while pathogens and other toxins need to be kept away from the blood stream.

1.3.1 Structure and function.

The intestine wall is divided into four layers (see figure 1) with mucosa being the first layer which is in direct contact with the ingested food (chymus), making it the first protective barrier. The mucosa consists of three supportive tissue layers, starting with specialized epithelia cells for absorption and protection supported by a connective tissue called lamina propria, which is filled with blood vessels, lymphoid tissue and nerves, and lastly a smooth muscle layer called muscularis mucosa, which can contract to change the shape of the wall locally (Turner, 2009). Protection for the GIT is accomplished though the physical epithelial mucosal barrier and the immune system referred to as gut-associated lymphoid tissue (GALT). GALT

consists of organized lymphoid tissue called Peyers patches and mesenteric lymphoid nodes which are main inductive sites to trigger an immune response, while the lamina propria which is a part of the mucosal layer and the epithelial layer is an effector site (Ahluwalia, Magnusson, & Öhman, 2017).

Submucosa is the second layer and consists of fibrous connective tissue, nerves and larger blood vessels, which transfer different molecules from the lumen to other organs and tissues in the body. Specialized nerve plexus called Meissner's plexus (submucosal plexus) makes up the first part of the enteric nervous system (ENS).

The third layer is called muscularis externa and consists of one inner circular and one outer longitudinal layer of smooth muscle fibers with the second and final part of the ENS, the myenteric plexus or Auerbach plexus. Neural innervation controls the contraction of these muscles and, hence, the mechanical breakdown and peristalsis of the food within the lumen. Both innervation from the central nervous system and ENS can excite or inhibit these muscles.

The fourth layer is called serosa and is formed by fat and another layer of epithelial cells called mesothelium (Sand, Sjaastad, & Haug, 2014).

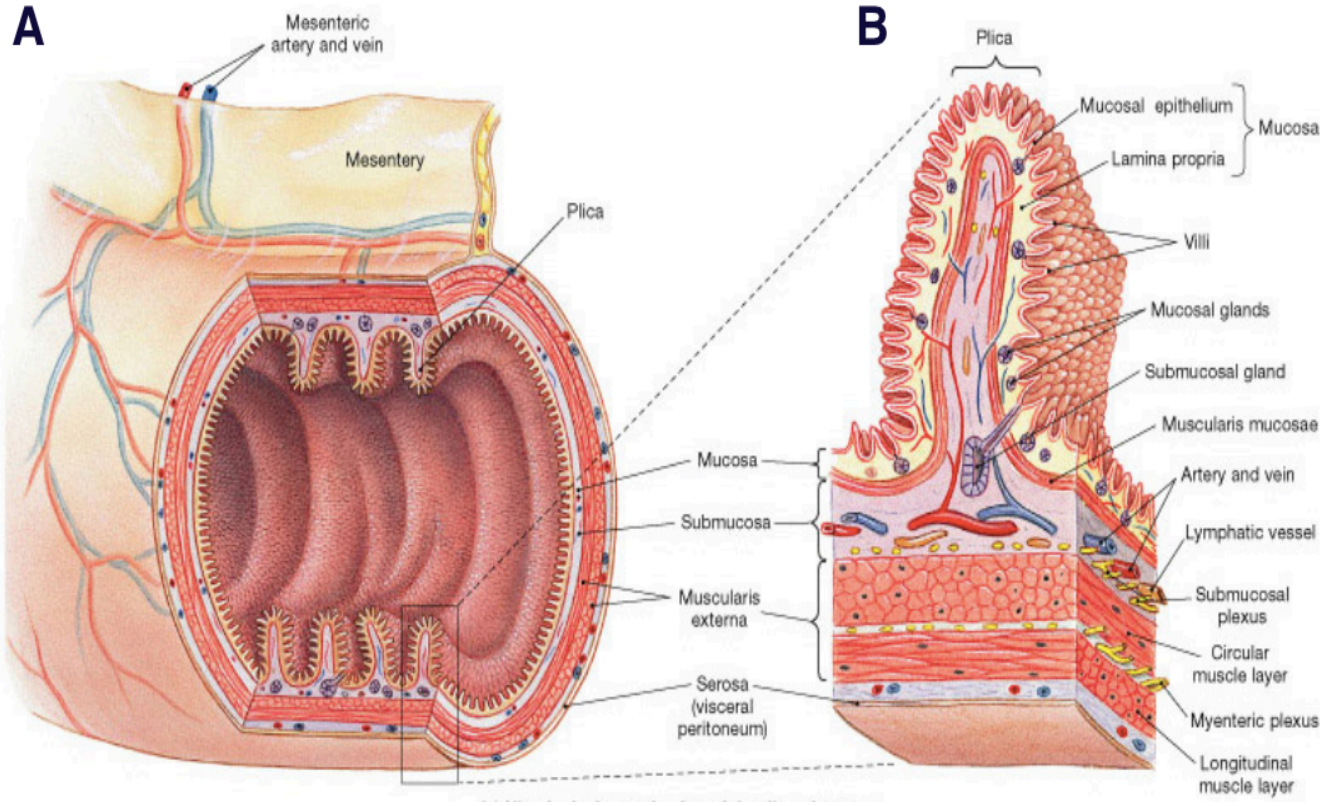


Figure 1. Structure of intestine wall. Figure A illustrates the whole intestine wall. Figure B illustrates how intestine layers are organized inside the intestine wall starting with mucosa, submucosa, muscularis externa and serosa. Reprinted from Pearson Education Inc, publishing as Benjamin Cummings (2004). Retrieved from https://slideplayer.com/slide/10182964/.

1.3.2 Small intestine. The biggest difference from the stomach to the intestine is that the lining of the intestine wall is covered by intestinal villi, which are extensions of lamina propria projecting into the lumen (see figure 1 B). The function of villi is to increase the internal surface area of the intestine walls, giving it a greater surface area for absorption, as they are connected to bigger blood vessels in the wall where the circulating blood carries these nutrients away. The mucosa of the small intestine contains several specialized cells, enterocytes are the most numerous and involved in nutrient absorption; enteroendocrine cells: secrete hormones such as secretin, cholecystokinin

among others; Microfold cells: sample antigens to the lymphoid tissue and are connected to; Paneth cells: secret lysozyme, a-defensin, bactericide and growth factors; Tuft cells; involved in the immune response; and Goblet cells: secrete mucus. Thus, some cells are responsible for absorption, whilst others secrete digestive enzymes and mucous to protect the intestinal lining. The cells are frequently replaced to ensure optimal intestinal barrier function (protection).

Duodenum is the first part of the small intestine, here the food from the stomach is neutralized, and where most of the chemical breakdown of nutrients to smaller molecules by enzymes takes place. Jejunum is the middle section and where most of the absorption of nutrients takes place, which is why the villi here are covered in a greater number of microvilli - smaller projections on top of the villi. The transport of nutrients across epithelial cells through the jejunum includes passive transport of some carbohydrates, and active transport of amino acid, peptides, vitamins and glucose. The ileum is the final section of the small intestine and its function is mainly to absorb any products of the chymus that were not absorbed by the jejunum. It also contains Peyer´s patches in the wall (lymphoid tissue rich with T- and B- lymphocyte immune cells).

1.3.3 Large intestine. Large intestine consists of the appendix, cecum, colon and rectum. The mucosa of the large intestine lacks villi and the mucosal surface is flat with several deep intestinal glands and goblets cells that secrete mucous to lubricate feces. The most important function of the large intestine is accumulation of unabsorbed material to form feces, digestion by bacteria and absorption of vitamins and reabsorption of water and salts. In contrast to the small intestine the large intestine has three separate parallel muscle tissues on the outer layer instead of one longitudinal muscle layer (Bharucha & Brookes, 2018). Under normal circumstances the bacteria constitute 1/3 of the feces

(Louis, Scott, Duncan, & Flint, 2007). They have the ability to digest cellulose and other complex carbohydrates that the body's enzymes can´t (McNeil, 1984). They can also synthesize some vitamins such as vitamin K and B, which can be absorbed by the large intestine. Another important function of intestinal bacteria is to shape and direct the development of the immune system through a mutual communication (Macpherson & Harris, 2004). The number of bacteria in the large intestine varies a lot, increasing when one is eating fiber (cellulose), or if digestion of proteins or carbohydrates in the small intestines does not function as it should. Treatment with antibiotics on the other hand reduces the number of intestinal bacteria (Garrett, Gordon, & Glimcher, 2010).

Contractions in the small and large intestine are based upon Cajal-cells which work as pacemakers. They have regularly spontaneous slow fluctuations in their membrane potential, which propagate by open cell-connections from one muscle cell to the next, resulting in a synchronous electrical activity in the muscle cells. The Cajal cells have alternating periods of rest and activity and different anatomical placement along the colon (Sanders, Koh, & Ward, 2006). The chymus will move towards the rectum with a speed of 5-10cm per hour in humans. If the passage takes longer time than usual, more water will be absorbed and constipation might occur; the feces becomes dry and hard resulting in problems with emptying which also can be painful. On the other hand, if the passage is too fast, not enough water will be absorbed causing diarrhea and dehydration. Constipation is normally due to weaker than normal contractions and movement in the large intestine, and can also be affected by emotional state (Tache, Martinez, Wang, & Million, 2004; Welgan, Meshkinpour, & Beeler, 1988). Diarrhea is often a result of the body trying to remove or prevent pathogens from sticking to the intestinal wall and cause harm (Binder, 2006).

1.4 Circadian rhythms in the gastrointestinal tract

The first evidence for involvement of the circadian clock in the gastrointestinal tract (GIT) came from observation in rodents and humans that epithelial cell proliferation, migration, differentiation and apoptosis oscillate as a function of time of day, measured by DNA synthesis. Peak in this synthesis is observed in a craniocaudal manner (first in duodenum, last in colon) – which is related to nutrient ingestion and transit, but importantly, persists during fasting (Bjarnason & Jordan, 2002; Scheving, 2000). This means that it is not driven by food ingestion, but by other mechanisms to anticipate food intake. This suggests that the protective epithelial barrier varies according to the time of day and different physiological needs. Another mechanism which is closely related to food intake, but also persists under period of fasting and variates throughout the day, is motility (contraction of intestinal muscles to drive food forward in the intestine). An increase in motility is observed after a meal and in the beginning of the active phase, with low activity in the rest phase (Karaus & Wienbeck, 1991).

Thus, since the GIT seems to be able to anticipate when food needs to be transported, nutrient absorption should also be anticipated to ensure efficient breakdown and uptake of nutrients in the active phase, balancing the high energy expenditure. Indeed, small intestine nutrient absorption seems to follow a circadian rhythm as Na^+/glucose transporter (SGTL1) which is involved in transporting glucose across the cell membrane, shows a higher expression during the active phase in mice, compared to their rest phase (Iwashina, Mochizuki, Inamochi, & Goda, 2011; Tavakkolizadeh et al., 2001). Also, H+ peptide co-transporter which transports peptides across the membrane has been proposed to be rhythmically expressed similar to the SGLT1 (X. Pan & Hussain, 2009). This seems to be consistent with how blood glucose homeostasis is maintained, with glucose uptake mainly from nutrients during the active phase and glucose release from storage (glycolysis) during the rest phase (Kalsbeek, la Fleur, & Fliers, 2014). Hence, the main function of the GIT, nutrient absorption, which is a complex process

involving multiple mechanisms, has at least some components showing circadian rhythmicity. This may also be the case for immune homeostasis, the second main function of the GIT, as Froy and Chapnik (2007) first found defensins (antimicrobial peptides) to be rhythmically expressed in the intestine crypts, but also that Paneth cells in mouse small intestine express toll-like receptors (TLRs) in a circadian manner (which in the GIT are one of the main mechanisms to combat pathogens). Thus, the physiological processes in the GIT are matched to temporal needs, implying that these processes are under circadian clock control.

1.4.1 The role of clock genes in the gastrointestinal tract. It has been established that clock genes are rhythmically expressed in oral GIT in humans (Bjarnason & Jordan, 2002), rat colon (Sládek et al., 2007) and in the stomach and colon of mice (Hoogerwerf et al., 2007). These clock genes were found to be functional as they maintained their rhythm in constant darkness, and their rhythm could be shifted according to feeding schedule, which has been demonstrated with other peripheral clocks (Damiola et al., 2000). Moreover, with the use of gene array studies, approximately 3.7% of colonic genes in mice were found to be under circadian control, most of them being involved in cell signaling, growth, cell proliferation and apoptosis (Hoogerwerf et al., 2008). Furthermore, Hoogerwerf and colleagues proposed a model of circadian regulation of colonic motility based on the findings of 1) rhythmic expression of clock gene *Per2* in colonic neurons of myenteric plexus, and 2) an absence of colonic motility rhythm in mice with a knock-out of the period clock genes. According to this model clock genes within neurons of the myenteric plexus modulate motility through direct and indirect clock driven transcription of genes involved in contraction of smooth muscle in the intestinal wall (Hoogerwerf et al., 2009).

As described above, the GIT consists of separate parts, each harboring functional peripheral circadian clock. In a study by Polidarová et al. (2009) clock gene expression was found to be phase-advanced in the upper part (duodenum) compared to the lower part (colon). See figure 2. It was also found that cell cycle checkpoint gene *Wee1* mirrored the same phase in each segment, which the authors claim supports the hypothesis that circadian clock controls the cycle of cell division in the intestinal mucosa of the GIT. Moreover, it also seems that clock genes in organs and tissues that have a central role in nutrient absorption and digestion, such as the liver and duodenum, are more easily affected by a feeding regime, compared to the colon. This was demonstrated in a study where rats that were kept under constant light for 30 days (no entrainment from SCN) and fed *ad libitium* (no food entrainment) had a loss of rhythmicity in clock genes in liver, duodenum and colon. A sub-group of rats exposed to restricted feeding for the last 15 days, had restored their rhythm in all clock genes, with the exception of the colon where only partial restoration was observed since *Per2* and *Wee1* were not rhythmically expressed (Polidarová, Sládek, Soták, Pácha, & Sumová, 2011).

The positive transcriptional activators of the primary feedback loop, the clock genes *Bmal1* and *Clock*, have been proposed to be involved in several functions in the GIT. *Bmal1* is suggested to be the driving element behind the circadian variation of the glucose transporter (SGTL1) since it has the ability to bind to the promoter of this gene, suggesting a central role for the primary clock genes (Iwashina et al., 2011; Tavakkolizadeh et al., 2001). Also, the BMAL1/CLOCK complex seems to regulate tight-junction proteins occludin and claudin, which regulate the permeability in colon epithelium. First, a time of day variation in expression of these tight-junction proteins was demonstrated. Second, mice who had a knock-out of *Per2* gene expressed chronic high levels of tight-junction proteins, while *Clock* knock-out mice expressed low levels

(Oh-oka et al., 2014). In addition, *Bmal1* also seems to be involved in the regeneration of the epithelial cell membrane after tissue damage, as mice lacking this gene had a disruption in both the circadian clock and rhythmic proliferation (Stokes et al., 2017). The authors also suggested that immune system cytokines were under circadian control, since they started to being rhythmically expressed during the damage. Thus, clock genes are important to maintain normal function, but also, perhaps even more important during challenging conditions. In summary, clock genes are rhythmically expressed along the GIT, but how clock genes contribute to different functions has only recently been studied and is yet to be fully understood.

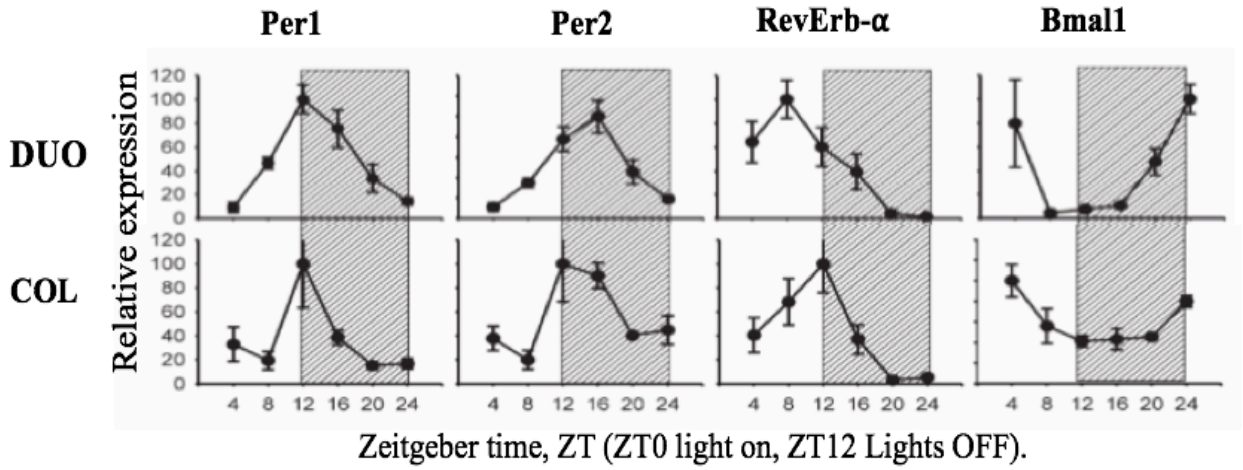


Figure 2. Circadian profile of clock gene expression of within different parts of rat intestinal mucosa (duodenum DUO, and colon COL). Expression of clock genes was determined every 4 h during the circadian cycle. Each data point represents the mean and SEM of three animals. Adapted from "Temporal Gradient in the Clock Gene and Cell-Cycle Checkpoint Kinase Wee1 Expression along the Gut" by L. Polidarovà, 2009, *Chronobiology International*, 26 (4), 607-620. (Polidarová et al., 2009)

1.5 Health consequences associated with shift work

1.5.1 Epidemiological studies. There are a number of different studies reporting an association between shift work and negative health outcomes such as sleep problems, metabolic dysfunction, cardiovascular disease and even cancer (Åkerstedt, 2003; Cappuccio et al., 2008; Karlsson, Knutsson, & Lindahl, 2001; A. Pan, Schernhammer, Sun, & Hu, 2011). In addition, there are studies that implicate that immune regulation is affected in shift workers (de Almeida & Malheiro, 2016). One topic that has been consistently reported to be negatively affected by shift work, but not received much attention in terms of experimental studies is the gastrointestinal tract function. In 2010 Knutsson & Bøggild did a systematic review of studies involving shift work and all types of gastrointestinal disease. Their result showed that 4 out of 6 studies found a significant association between shift work and gastrointestinal complaints such as reduced appetite, abdominal pain and bowel discomfort, while 5 of 6 studies reported an association between shift work and peptic ulcer. A few studies at that time investigated chronic inflammatory bowel diseases (Chron´s or ulcerative colitis) or cancer in relation to shift work, but were inconclusive in establishing an association.

In a more recent meta-analysis one group of scientists found that night shift work is correlated with an increased risk for colorectal cancer, with the ratio increasing with 11% for each 5th year on a night shift work schedule (X. Wang et al., 2015). Furthermore, a higher prevalence of irritable bowel syndrome was found among a cohort of nurses who worked rotating shifts compared to nurses on day shift only (Nojkov, Rubenstein, Chey, & Hoogerwerf, 2010). In sum, these epidemiological studies seem to associate night shift work with negative short-term effects and a risk for developing more severe long-term disturbances in the gastrointestinal tract which deserves

investigation. To date, no experimental studies on human shift work and gastrointestinal function exist.

1.5.2 Experimental studies. There are no experimental studies on human shift work and gastrointestinal functions, but there are some in other tissues, which might have implications for the gastrointestinal tract. Shift workers who are affected by sleep debt or insomnia often display an elevated response to stress and a deregulation of the hypothalamus-pituitary-adrenal axis and its mediator cortisol (Meerlo, Sgoifo, & Suchecki, 2008). In an early study on the effect of phase shift on cortisol levels, male participants had their sleep/wake cycle shifted with sleep time from 07:00 to 15:00 for two consecutive days. After the second day a new peak was observed in cortisol levels at 14:00, compared to 07:00 in controls (Goichot et al., 1998). This finding is further supported by other studies where a reduction in mean amplitude of the cortisol rhythm is generally seen following night shift work (Lac & Chamoux, 2004). It is well known that cortisol has a strong anti-inflammatory effect on the immune system (Barnes, Adcock, Spedding, & Vanhoutte, 1993) and is likely responsible for decreasing immune cells in the beginning of the active phase both in human and rats, as cortisol levels rise; thus, making components of the immune system to oscillate over the course of a day. Circadian variation is observed in plasma proinflammatory type 1 cytokines (Interleukin IL-2, IFNγ and IL-12) and anti-inflammatory type 2 cytokines (IL-4, IL-10). Anti-inflammatory type 1 is dominating in the rest phase (night) and proinflammatory type 2 dominating throughout the active phase (day) in healthy humans (Haus & Smolensky, 2013). It is therefore reasonable to speculate that a reduction in cortisol levels in shift workers in the morning might affect this balance. In line with this, one study that investigated the proinflammatory cytokine profile in saliva in day- and night-workers,

found altered rhythmicity in tumor necrosis factor (TNF), IL-1β and IL-6 in the night shift workers (Reinhardt, Fernandes, Markus, & Fischer, 2018).

Furthermore, an increase in proinflammatory activity can lead to an increase in oxidative stress. Oxidative stress is a result of oxygen producing changes in our bodies causing large chain chemical reactions known as oxidation. Antioxidants are compounds that can prevent oxidation. Oxidative stress is due to imbalance between antioxidants and oxidants, with higher levels of oxidation, which can cause damage to proteins, lipid membrane, DNA, and ultimately cause cell death (Bandyopadhyay, Das, & Banerjee, 1999). The immune system uses oxidants in a beneficial way to kill pathogens during an onset of an immune response. This is accomplished when phagocytes increase their oxygen uptake as much as 10-20 folds causing a respiratory burst and creating reactive oxidative species, which in turn facilitates phagocytosis during an immune challenge (Rahal et al., 2014). However, if this immune response is not regulated it may ultimately injure the host tissue since these reactive oxygen species are non-specific. This process can also be reversed, where oxidative stress triggers or hyperactivates an immune response (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014).

Glutathione peroxidase-1 (GSH-Px) is most abundant intracellular antioxidant enzyme version that is found in the cytoplasm of nearly all mammalian tissue (Lubos, Loscalzo, & Handy, 2011). It is a natural antioxidant that is currently considered to be one of the most important biomarkers for oxidative stress (Rahal et al., 2014; Singh et al., 2005). In humans and rodents there is a circadian time-of-day variation in plasma GSH-Px levels, with the lowest values in the inactive phase and a peak in the middle of the active phase (Cao et al., 2015; Singh et al., 2005). In addition, the timing of the rhythm seems to be approximately the same across different tissues, as values peak late in the active phase both in blood, liver and brain tissue (Baydas et al., 2002).

A human study using one night of total sleep deprivation as a model of night shift work found increased expression of interleukin 1 receptor complex (binding site for IL-α and IL-β), involved in pro-inflammation and increased expression of GSH-Px in tissue samples from fat and muscle, suggesting an increase in inflammation and oxidative stress across tissue following sleep loss (Cedernaes et al., 2018). In a more recent study, Teixeira et al. (2019) used a real-life situation where they compared males which had a permanent long-term schedule on either day shifts or night shifts. They found higher blood levels of markers of oxidative stress and GSH-Px – marker of antioxidant defense in the night workers, indicating an unsuccessful removal of oxidants with the result of oxidative stress. Furthermore, they argue that this is the first study to show a chronic effect of night shift work and oxidative damage. One important aspect was that the results were independent of social-jet lag which is a measure of sleep debt. Thus, in the experiment by Cedernaes et al. (2018) the increase in inflammation and oxidative stress might be due to change in timing of activity (being awake during the rest phase) and not due to sleep debt per se. If similar changes can be observed in the gastrointestinal tract after night shift work remains to be determined.

1.6 Animal studies modelling shift work in humans

Animal studies make it possible to more closely investigate the mechanistic effects behind the association between night shift work and health consequences found in human studies. Moreover, the use of animal models allows for easier randomization and control of variables. Rodents (mice and rats) which are often used in these experiments have a similar circadian rhythm as humans, but with a different timing. Rodents are nocturnal animals, meaning their active phase is at night and their rest phase at daytime. By manipulating their surroundings and changing their rest/active cycle one can mimic human night shift work. This

can be performed by using different techniques; forcing the animals to be awake during their rest phase, restricting food intake, preventing sleep in their rest phase or changing the light/dark cycle (Opperhuizen, van Kerkhof, Proper, Rodenburg, & Kalsbeek, 2015).

Bergen Stress and Sleep Group has in the recent years established a rat model of simulated night shift work (Grønli et al., 2017). This model uses forced activity in either the rest phase to mimic night shift work or in the active phase to mimic day shift work. Results from this model show that three consecutive night shifts lead to short-term and enduring sleep and electroencephalographic disturbances, which is compatible with human findings of disturbed sleep and degraded wakefulness (Grønli et al., 2017). Three consecutive night shifts also led to changes in time-of-day variation of BMAL1-driven translational activity (regulation of protein synthesis) in rat prefrontal cortex (Marti et al., 2017). Moreover, altered metabolic gene expression in the rat liver and changes in the timing of food intake was found after simulated night shift work compared to day shift (Marti et al., 2016). Preliminary results showed altered clock gene expression (upregulation of *Bmal1* and *Clock* and downregulation of *Per2* and *RevErb-α*) in the liver tissue along with increased expression of the proinflammatory interleukin IL-α and IL-β (unpublished results). These findings are in line with others that find adverse negative metabolic consequences after simulated night shift work (Opperhuizen et al., 2015). The effect of this model on the gastrointestinal tract and its functions has yet to be elucidated.

1.6.1 Studies on circadian rhythm disruption and gastrointestinal function. The closest experimental studies to simulate night shift work in relation to gastrointestinal function are studies using different circadian disruption regime. An early study by Preuss et al. (2008) used chronic circadian disruption and exposed mice to a continuous 12h phase shift of the light/dark cycle every 5th day for three months, and investigated the severity

of experimentally induced colitis by dextran sodium sulfate (DSS). The phase shifted mice developed a more severe form of colitis that was associated with more significant weight loss and overall higher mortality compared to mice with a normal circadian rhythm. Implication of this finding is that colonic epithelial mucosa is highly sensitive to circadian disruption when the immune system is challenged by DSS. This is further supported by another study using the same chronic circadian disruption where increased permeability of the intestinal epithelia barrier was found (Summa et al., 2013). In another study by Polidarová, Houdek, and Sumová (2017), the authors wanted to see if circadian disruption can affect not only a challenged immune system, as Preuss group did, but also affect the unchallenged immune state. One group of rats were phase advanced by 6 h and phase delayed back again after 2 days for a total of 16 days, while another group had constant light for 4 weeks where the lights were not turned off for the entire period. They found an upregulation of *IL-α* and a downregulation of anti-inflammatory marker *Rgs16* in the phase advance/delay condition, but with a more pronounced effect in constant light condition where multiple pro-inflammatory markers were upregulated including *IL-α.* Furthermore, the results were independent of sleep deprivation, as measured through activity/rest cycle. Thus, it seems that maintenance of a protective intestinal epithelial barrier is strongly affected by circadian organization, which might be a possible mechanism behind increased inflammatory response seen in circadian disruption studies.

1.7 Aims and hypothesis

This study was conducted to assess effects of three consecutive days of simulated shift work on gastrointestinal function in rats. The first main aim of this study was to measure the time-of-day variation in 1) protein marker of oxidative stress (glutathione peroxidase 1, GSH-

Px) and proinflammatory processes (Il-α and calprotectin) in feces, and 2) characterize clock gene expression (*Bmal1, Clock, Per1, Per2,* and *RevErb-α)* in intestinal mucosa, and measure expression of other candidate genes involved in proinflammatory processes (Il-α); glucocorticoid receptor activity (*NR3C1*); oxidative stress marker (glutathione peroxidase, *GSH-Px*) and cell cycle checkpoint (*Wee1*) in the same tissue. The second main aim was to investigate the extent to which one simulated shift work period affects these dynamic processes on protein marker in feces and gene expression in intestinal mucosa.

I hypothesized to find:

1) Time-of-day variation:
    a) Decreased fecal levels of GSH-Px1 in transition to active phase (defined as zeitgeber time 12 (ZT12), when lights are turned OFF) compared to transition to rest phase (defined as zeitgeber time 0 (ZT0), when lights are turned ON).
    b) No difference in fecal levels of IL-α and calprotectin in transition to active phase (ZT12), compared to transition to rest phase (ZT0)
    c) Specific time of day variation in expression of clock genes in both duodenum and colon tissue; downregulation of genes in the positive loop (*Bmal1 and Clock*) and upregulation of genes in the negative loop (*Per1, Per2 and RevErb-α)* in transition to active phase (ZT12) compared to transition to the inactive phase (ZT0).
    d) Decreased expression of glucocorticoid receptor (NR3C1) in transition to active phase (ZT12) compared to transition to inactive phase (ZT0).

2) Effect of one shift work period:
    a) Increased fecal levels of GSH-Px in rest phase workers relative to their time-matched controls.

b) Increased fecal levels of IL-α and calprotectin in rest phase workers relative to their time-matched controls.

c) Altered gene expression of clock genes and other candidate genes in rest phase workers (simulated night shift work) compared to time-matched controls.

2 Methods

2.1 Ethical approval

The project was approved by the Norwegian Animal Research Authority (permit number: 11321) and conducted in compliance with the Norwegian laws and regulations controlling experiments in live animals and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

2.2 Animals and housing

Male rats (n= 32, Sprauge Dawley, NTac:WH, Taconic, Denmark) weighing approximately 300g upon arrival were group housed in individually ventilated cages (IVC, Techniplast, Italy, 75 air changes per hour) type IV (480 x 375 x 210mm, 1500cm^2), in standard conditions (controlled temperature room 23-25° C; humidity 40-50%). After 5 days of acclimatization animals were housed individually (IVC cage type III, 425 x 266 x 185mm, 800cm^2) and randomized into different groups. Food (rat and mouse no. 1 (RM1), Special Diets Services, Witham, Essex, England) and filtered water was provided *ad libitum* before and throughout the experiment. Cage bedding (BK bedding, Scanbur BK) was changed weekly, except during the course of the experiment. Animals were handled by trained and certified personnel. Appropriate clothing and equipment were worn at all times.

The animals were maintained on a normal 12 h light/12 h dark (LD) schedule. Lights went on at 08:00, this time point was defined as zeitgeber time 0 (ZT0), and lights went off at 20:00, defined as ZT12. Lights were gradually dimmed on and off over a period of 1 h fully on at 9am, and fully off at 21:00.

2.3 Experimental Design

The experimental design is an independent group design consisting of four (five for feces) different groups. The design comprised 3 consecutive work days constituting one period of shift work, followed by tissue and feces collection two hours after the ended work shift on the 3rd work day. See figure 3.

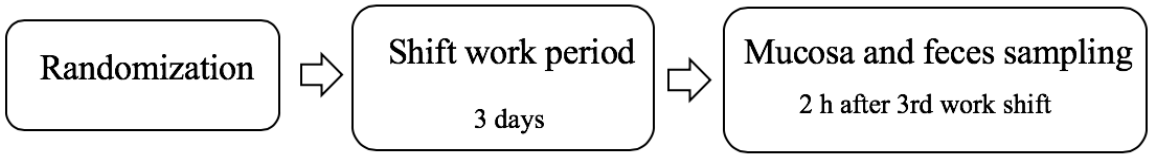


Figure 3 . Timeline for experimental protocol.

For the experimental condition animals were randomly assigned to either rest phase work (RW, n=9) or active phase work (AW, n=7). Another batch of animals served as undisturbed controls, see figure 4. In order for them to act as controls to the experimental groups they had to be sacrificed at the same zeitgeber time. Therefore, these animals were randomly assigned to either rest work control (RWC, n=8), or active work control (AWC, n=8). After the shift work period fecal pellets were collected and stored, until it was incorporated and used in this design, as an additional intervention (see figure 4).

In 2017 Bergen Stress and Sleep group performed an experiment with the same experimental protocol, and in addition had one group of rest phase workers who worked in blue-enriched light (RWB, n=9). The purpose at that time was to look at changes in brain

activity, and not in relation to the gastrointestinal tract. In this project fecal pellets from the group will serve as explorative research and added as an additional intervention (see figure 4).

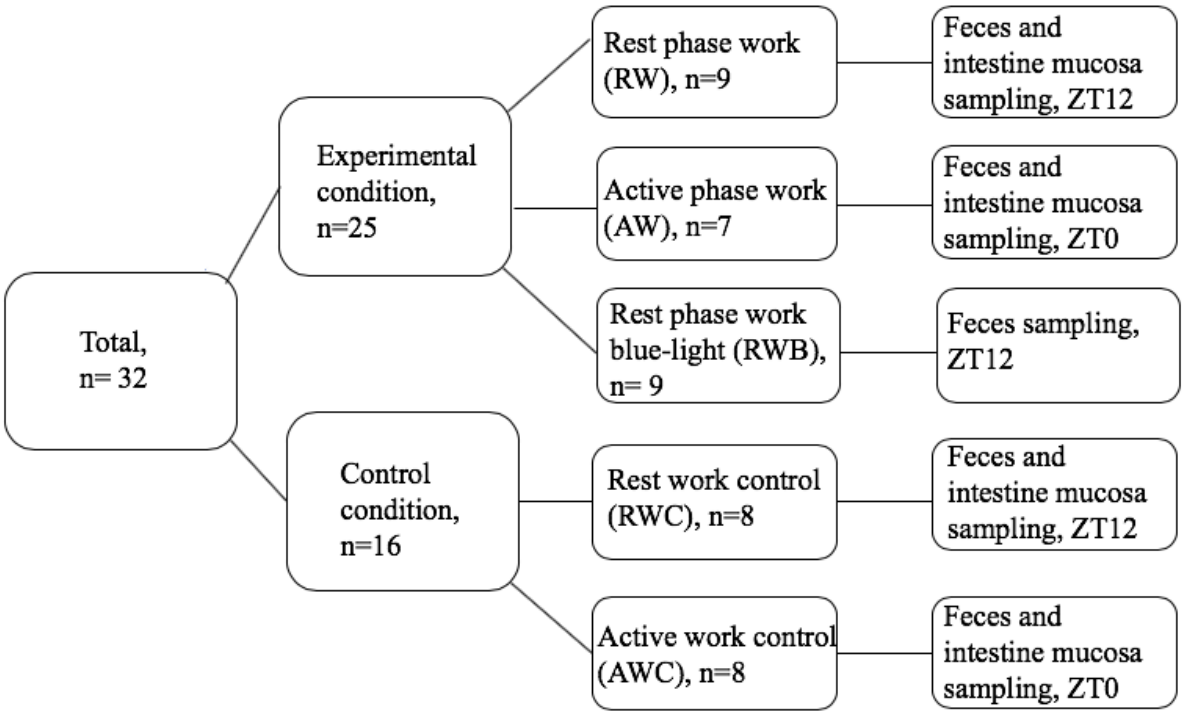


Figure 4. Assignment of animals into experimental condition (n=25) and control condition (n=16). Feces and intestine mucosa were collected at the end of the active phase at zeitgeber time 0 (ZT0, lights ON) for active phase workers (AW) and active phase worker controls (AWC), and at the end of the rest phase at zeitgeber time 12 (ZT12, lights OFF) for rest phase workers (RW), rest phase workers in blue light (RWB) and rest phase worker controls (RWC).

2.4 Simulated shift work procedure

To mimic one period of simulated shift work in humans, rats underwent sessions of enforced ambulation for 8 hours in three consecutive days. One group of animals worked in their active phase (ZT14-ZT22, active workers, AW) at the circadian time when the animal is physiologically primed for wakefulness, thus simulating day shift work. Another group of animals worked in their rest phase (ZT2-ZT10, rest workers, RW); hence by keeping the animal

awake at the time of day when the animal is primed for sleep, night shift work is simulated. See figure 5. The motorized running wheels (TSE, Germany) used for enforced ambulation, have a diameter of 24cm and are set to 3rpm, which forces the rats to stay awake and to move slowly while allowing them to eat and drink *ad libitum.* After the time in the wheels, the rats were returned to their home cages with food and water available *ad libitum* and left undisturbed until the next workday.

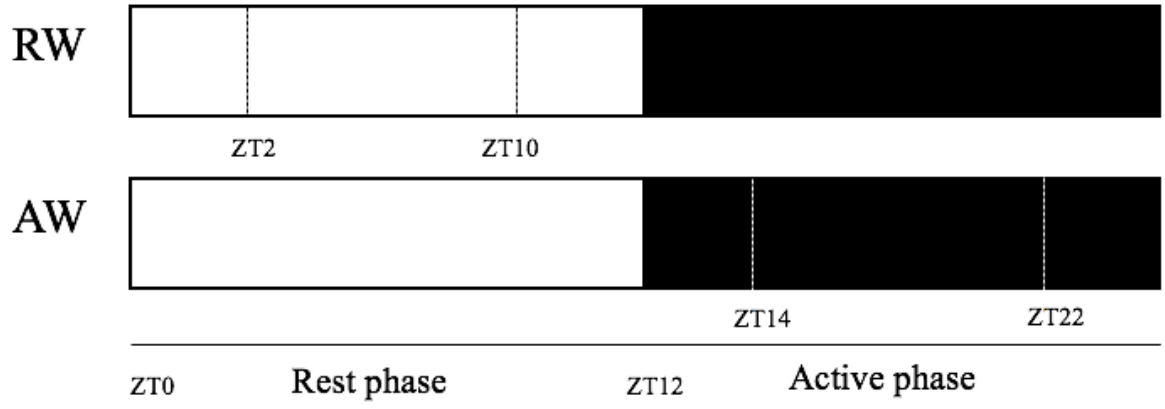


Figure 5. Shift work schedule. Time for lights ON is defined as zeitgeber time 0 (ZT0), and lights OFF as ZT12. Dashed lines represent the start and end of enforced ambulation (work). Rest workers (RW) started working at ZT2 and finished at ZT10. Active workers (AW) started working at ZT14 and finished at ZT22. Both groups were left undisturbed in their home cage until the next work day. One shift work period lasted for 3 consecutive days.

2.5 Sample collection of feces and intestinal mucosa

Following the third work session, animals were placed in their home cage for 2h, and left undisturbed. Subsequently, they were anesthetized with isoflurane (Isobavet, Schering-Plough), and sacrificed by decapitation. Intestines were dissected out for collection of feces samples and samples of intestinal mucosa. The animals were sacrificed at different zeitgeber times: AW (n= 7) at ZT0 (light ON), and RW (n= 9) at ZT12 (lights OFF). The second batch of undisturbed animals which were never exposed to simulated shift work were sacrificed at

the same zeitgeber times as experimental animals (AWC, ZT0, n= 8) and (RWC, ZT12, n= 8). Data from these animals were used to investigate time-of-day variation (ZT0 vs. ZT12), and for comparison of data from their time matched experimental groups (AWC vs. AW, and RWC vs. RW). See figure 4

Feces collection from the third experimental group, rest phase work blue-enriched light (RWB), followed the same protocol as RW with sampling at ZT12. Data from these animals were used for comparison of data from their time matched controls (RWC) and rest phase workers in standard light (RW).

2.5.1 Feces sampling. Feces was sampled from colon post mortem. All feces-pellets available from rectum to 5cm from cecum were collected, average of 4-5 feces-pellets. See figure 6, C. All feces samples were collected in plastic bags and stored at -20°C until analysis.

2.5.2 Intestinal mucosa sampling. Mucosa samples were collected from the proximal part (relative to the stomach) of duodenum and colon. First, the whole intestine was carefully dissected with scissors and tweezers, removed from the animal and layered out after a template (see figure 6.) so each intestine section of interest could be located. Each segment was cut to a length of approximately 4 cm, opened up along the longitudinal muscle layer with a scissor and flushed with phosphate buffer. The mucosal layer was separated (scraped off) from the rest of the intestine layers with a glass slide and collected in Eppendorf tubes. The whole procedure from decapitation to collected mucosa lasted approximately 30 minutes. The samples were temporarily placed on dry ice before being stored at -80°C until analysis.

.

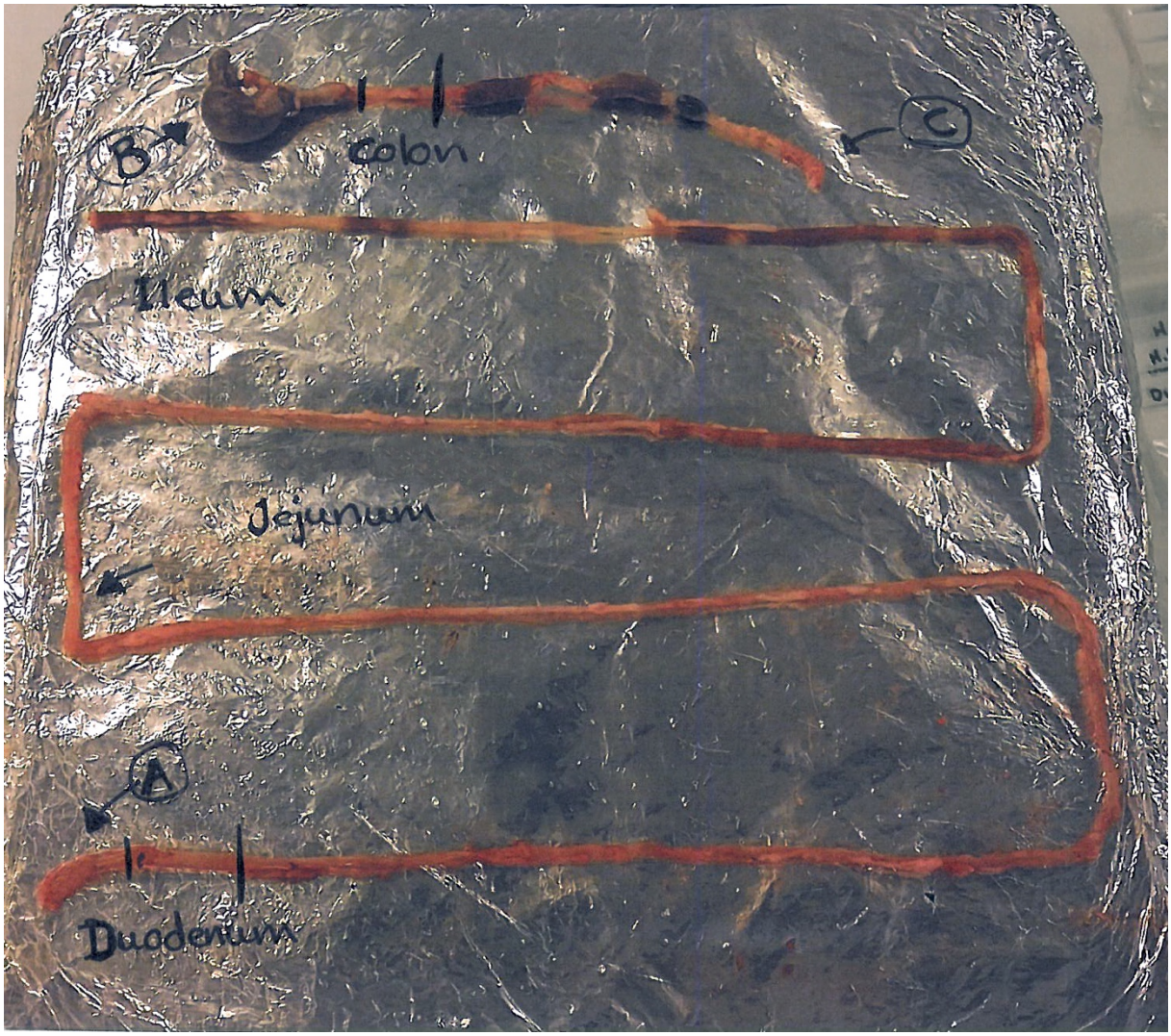


Figure 6. Rat intestine layered out showing A) proximal duodenum which is connected to the stomach, followed by the small intestine jejunum and ileum which connects to B) cecum and the large intestine (colon) and C) rectum. The vertical lines represent where the intestine was cut and mucosa collected. Feces were collected from C to first vertical line at colon.

2.6 Analyses of marker in feces and intestinal mucosa

2.6.1 Protein markers in feces. Assessment of protein markers in feces was performed using enzyme-linked immunosorbent assay (ELISA) technique.

2.6.1.1 Homogenization of feces pellets. All feces pellets from each animal were crushed in frozen form and mixed while still in plastic bag. Then for each sample 100 mg of feces was weighted and used in each analysis. For calprotectin analysis feces samples were homogenized by diluting 100 mg feces in 5 ml extraction buffer and centrifuged for 10 minutes at 3000 g. For IL-1α and GSH-PX feces was homogenized by diluting 100 mg feces in 100 ml phosphate buffer and centrifuged for 20 min at 3000 g before being assayed.

Before assay the individual samples were randomized to different places on the analysis kit to ensure that no experimenter bias would occur and to ensure that any potential manufacturing differences in the kit wells would not affect an experimental group as a whole. Assay was performed according to the instruction manual for each analysis kit, and optical density quantified by ELISA microplate reader (Molecular Devices, SpectraMAX plus 384, Softmax Pro 5.4.5).

2.6.1.2 Enzyme-linked immunosorbent assay (ELISA). ELISA is a sensitive method which detects the presence of a biological substance of interest based on antigen-antibody interaction. Sandwich ELISA is a technique which detects antigen. Feces samples were analyzed using ELISA for detection of proinflammatory marker IL-1α (Rat Interleukin 1 α ELISA kit, CUSABIO BIOTECH CO) and oxidative stress marker glutathione peroxidase 1 (Rat Glutathione Peroxidase (GSH-PX), MyBioSource.com). Additional analysis was performed to quantify calprotectin (S100A8/S100A9 Calprotectin, MRP 8/14, Immundiagnostik AG) – a marker of inflammatory activity related to gastrointestinal disorders.

The procedure involves the following: a specific antibody is attached to a solid surface in the well, then the sample of interest, in this case homogenized feces, is added

for detection of antigen, in this case GSH-Px1, calprotectin and IL-α. If there is a presence of antigen they will bind to the antibodies. Afterwards an enzyme-linked antibody will be added for further binding to the antigen. Wash buffer is then added, so only the bound chain of antibody-antigen-enzyme substances are left in the well. Then a substrate is added which reacts with the enzyme and create a color reaction. The optical density of each sample is detected and compared to a standard curve, which is typically a serial dilution of known-concentration solution of the target molecule.

Recommended sample weight for feces was 0.1 g, however this number was difficult to reach exactly given the material composition (resulting in weight either above or below 0.1 g). Therefore, the actual weight for each sample was recorded beforehand and after assay the results was corrected for weight. For calprotectin and IL-α result was multiplied with 0,1 since pipetting was 100 µl to each well, then divided by actual sample weight - giving a corrected result in ng/g. For GSH-PX, 50 µl was pipetted to each well and multiplied with 0,00005 L to get a corrected result in U/L (unit per liter) and divided by sample weight.

2.6.2 Mucosal tissue gene expression analysis. Real time-polymerase chain reaction (RT-PCR) is a method to investigate gene expression in a given tissue. In short, RNA must be isolated from the tissue before gene expression is assessed by RT-PCR.

2.6.2.1 Homogenization and RNA isolation of intestinal tissue. For RNA isolation RNeasy mini kit (Qiagen) was used according to the manufacturer's instruction. Each sample weight was recorded beforehand. First, lysis buffer was added to the mucosa samples, in order to prevent degradation by an enzyme called RNase, making the samples more stable. For homogenization the samples were exposed to a high-speed shaking with

beads (TissueLyser, Qiagen, USA), which grinds and beat the tissue to fully release biomolecules. Secondly, after homogenization the samples were pipetted to new filter-based tubes that utilize a membrane in the middle. When the lysate is passing though the membrane the nucleic acids sticks to the membrane by the use of centrifugal force. Wash solutions are subsequently passed through the membrane and discarded, before an elution solution is applied and the RNA sample is collected into a new tube by centrifugation.

For each sample RNA concentration and purity were then assessed by UV spectroscopy (NanoDrop 2000 Spectrophotometer, Thermo Scientific, USA). The absorbance of a diluted RNA sample is measured at wave lengths between 260 and 280 nm. Recommended nucleic acid (RNA) concentration for gene expression analysis is 20 ng/ul and above. In our sample one sample from duodenum tissue fell below the recommended value at 14,8 ng/ul. (For concentration values in each sample see appendix B.

2.6.2.2 Real-time polymerase chain reaction (RT-PCR). RNA extraction was first performed using 6100 Nucleic acid PrepStation (Applied Biosystem, USA). The next step involved RNA transcription to cDNA, using High Capacity RNA-to-cDNA kit (Applied Biosystems). RT-PCR was run on the Applied Biosystems 7900 Real-Time PCR System, with each sample run in triplicate. The method is based on fluorescence-detecting thermocyclers to amplify specific nucleic-acid sequences and measures their concentrations simultaneously. Relative gene expression levels were determined using the comparative ΔCt method, using β-actin (Actb), ribosomal protein lateral stalk subunit P0 (Rplp0) and b2-microglobulin (B2M) as endogenous controls. These genes were chosen as previous studies have used them and found their expression to be stable

throughout 24 h, and did not vary markedly between analyzed tissue in rat liver, duodenum and colon (Marti et al., 2016; Polidarová et al., 2017; Polidarová et al., 2009). Furthermore, geometric mean of the control genes was calculated using the formula: $\left(\prod_{i=1}^{N} x_i\right)^{1/N} = \sqrt[n]{a_1 a_2 \cdots a_n}$

Results are given as relative expression values normalized to the calculate geometric mean to ensure accurate normalization of RT-PCT data, as recommended by Vandesompele et al. (2002).

2.7 Statistical analyses

Statistical analyses were conducted using Statistica™ (version 13.3, TIBCO® Software Inc). Experimental group (AW, AWC, RW, RWC, RWB) was classified as dependent variable and effects of time-of-day dependent variation and shift work schedule were tested using one-way analysis of variance (ANOVA). Statistical significance was accepted at $p<0.05$. Cohens d was calculated for between group comparisons (M1-M2)/SD pooled) as an estimation of effect size. An effect size of 0.2 is considered small, around 0.5 is considered a medium effect and 0.8 and above is considered a large effect (Cohen, 1992). Positive d values represent an increase and negative values represent a decrease in one group relative to another. Outlier exclusion criteria for individual data points was set to 2 standard deviation from the group mean, that is why there are different degrees of freedom in the results section. A total of six animals were excluded based on this criteria. One animal from the rest-phase worker control group (RWC) was excluded from statistical analyses of *RevErb-α* expression in time-of-day variance and effect of shift work. Another animal from RWC was excluded from statistical analyses of *Clock* expression in time-of-day variance and effect of shift work. One animal from the active-phase worker control group (AWC), and one from the active-phase worker (AW), and one from rest

phase worker (RW) were excluded from statistical analyses of IL-α expression in time-of-day variance and effect of shift work. Another animal from AWC was excluded for statistical analyses of *Wee1* expression in time-of-day variance and effect of shift work. All results are presented as mean ± SD.

3 Results

3.1 Time-of-day dependent variation

3.1.1 Protein markers in feces. ELISA analysis of IL-α gave only detectable values for the following rats: AW2 (23.11 ng/ml), AWC10 (8.49 ng/ml), AWC12 (4.80 ng/ml), AWC8 (8.09 ng/mL), RW9 (1.90 ng/ml), RWB18 (10.70 ng/ml), RWC1 (1.19 ng/ml), RWC13 (3.76 ng/ml). The rest of the sample gave no values since they were outside the standard range of the standard curve (detection range 0.39 ng/ml -100 ng/ml). Therefore, no statistical analyses could be performed on this marker.

Significant time of day variance in GSH-PX levels ($F_{(1, 14)} = 7{,}95$ $p<0.01$.) GSH-PX levels were higher at ZT12 (RWC, 0.76 ± 0.06 U/L) compared to ZT0 (AWC, 0.61 ± 0.13 U/L), see figure 7 A. This difference showed a medium effect size ($d = 0.48$).

There was no significant time of day variance in calprotectin levels ($F_{(1, 14)} = 0.60$, $p>0.44$). Descriptively, calprotectin levels were higher at ZT12 (RWC, 5.26 ± 2.90 ng/g) compared to ZT0 (AWC, 4.40 ± 1.08 ng/g) with medium effect size ($d = 0.39$), see figure 7 B.

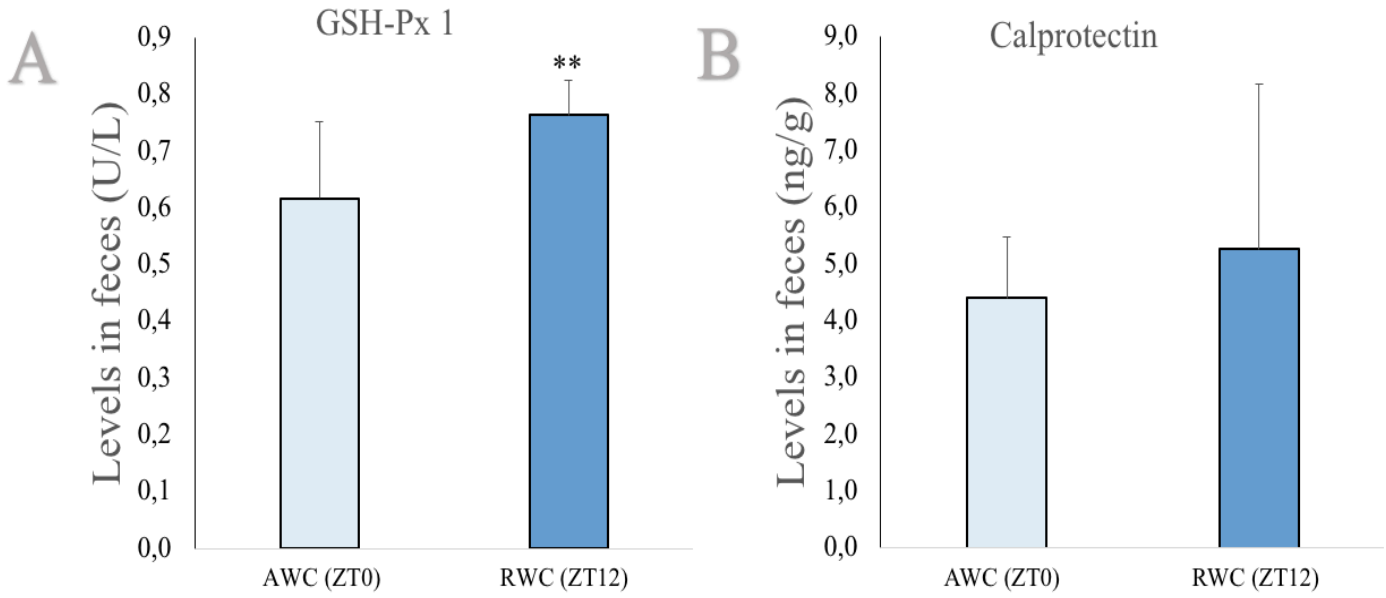


Figure 7. Protein markers in feces of A) oxidative stress marker (glutathione peroxidase 1, GSH-Px) and B) proinflammatory activity (calprotectin) in control animals: rest worker control (RWC, feces sampled at zeitgeber time ZT12) and active worker control (AWC, feces sampled at ZT0). Error bars represent standard deviation from the mean; **p<0.01. U/L abbreviation for units per liter.

3.1.2 mRNA expression in intestinal mucosal layer. RT-PCR analysis of duodenum tissue gave in general variable results, with low gene expression values and analysis was therefore considered as not reliable (see discussion). No statistical analyses could be performed using this segment of intestinal tissue. All reported gene expression results are from colon tissue.

3.1.2.1 Clock genes. There was a significant time of day variance in *Bmal1* expression ($F_{(1, 14)} = 298.44$, $p<0.001$). *Bmal1* levels were lower at ZT12 (RWC, 0.10 ± 0.02) compared to ZT0 (AWC, 1.00 ± 0.14). This difference showed a large effect size (d = -9.). See figure 8 A.

There was a significant time of day variance in *Clock* expression ($F_{(1, 13)} = 43.35$, $p<0.001$). *Clock* levels were lower at ZT12 (RWC, 0.66 ± 0.02) compared to ZT0 (AWC, 1.00 ± 0.13) This difference showed a large effect size (d = -3.65). See figure 8 B.

Per1 expression also showed a significant variance ($F_{(1,14)} = 57.71$, $p<0.001$). *Per1* levels were higher at ZT12 (RWC, 3.75 ± 0.97) compared to ZT0 (AWC, 1.03 ± 0.28). This difference showed a large effect size (d = 3.81). See figure 8 C.

There was no significant time of day variance in expression of *Per2* ($F_{(1, 14)} = 0.06$, $p>0.79$). Descriptively, *Per2* levels were higher at ZT12 (RWC, 1.21 ± 0.30) compared to ZT0 (AWC, 1.15 ± 0.62) with a small effect size (d = 0.12). See figure 8 D.

There was a significant time of day variance of *RevErb-α* expression ($F_{(1,13)} = 181.00$, $p<0.001$). *Reverb-α* levels were higher at ZT12 (RWC, 63.76 ± 10.50) compared to ZT0 (AWC, 1.33 ± 1.22). This difference showed a large effect size (d = 8.35). See figure 8 E.

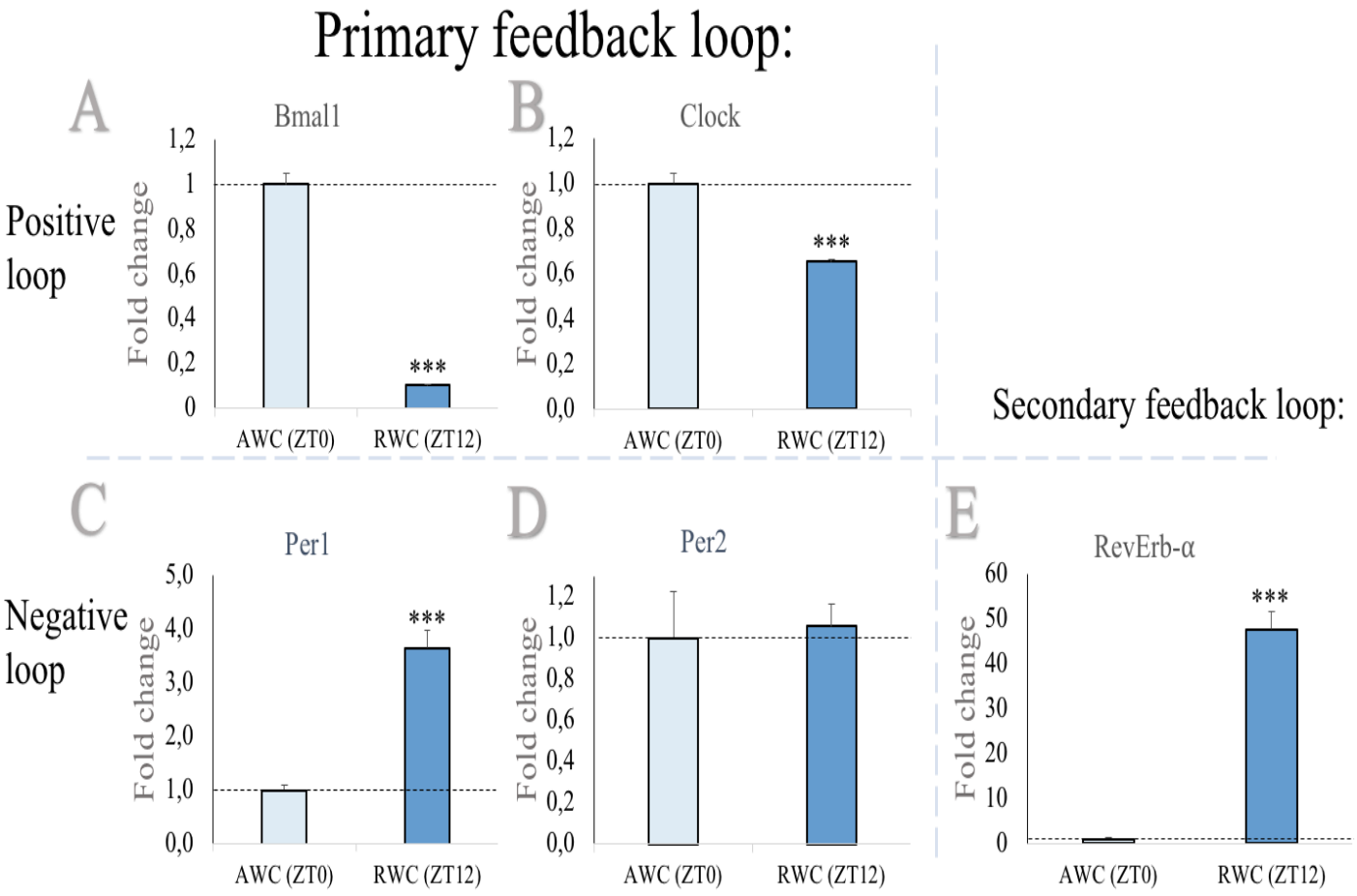


Figure 8. Clock gene expression in control animals: rest worker control (RWC, intestinal mucosa sampled at zeitgeber time 12) and active worker control (AWC, intestinal mucosa sampled at zeitgeber time 0) in the primary feedback loop: A) *Bmal1* and B) *Clock* (positive loop), and C) *Per1* and D) *Per2* (negative loop); and the secondary feedback loop: E) *RevErb-α.* Error bars represent standard deviation from the mean; *** $p<0.001$.

3.1.2.2 Other candidate genes. There was no significant time of day variance in expression of *Wee1*, marker of cell cycle checkpoint: ($F_{(1, 13)} = 0.29$, $p>0.59$). Descriptively, *Wee1* levels were lower at ZT12 (RWC, 0.92 ± 0.24) compared to ZT0 (AWC, 1.06 ± 0.44) with small effect size (d = -0.39). See figure 9 A.

Also, no significant time of day variance in expression of *GSH-PX,* marker of oxidative stress ($F_{(1, 14)} = 0.75$, $p>0.39$). Descriptively, *GSH-PX* levels were lower at

ZT12 (RWC, 0.93 ± 0.17) compared to ZT0 (AWC, 1.01 ± 0.17) with medium effect size (d = -0.47). See figure 9 B.

Again, no significant difference in time of day variance in gene expression of *NR3C1*, marker of glucocorticoid receptor activity ($F_{(1, 14)} = 0.77$, $p>0.39$). Descriptively, *NR3C1* levels were higher at ZT12 (RWC, 1.07 ± 0.09) compared to ZT0 (AWC, 1.01 ± 0. 18) with small effect size (d = 0.42). See figure 9 C.

Finally, there was no significant time of day variance in gene expression of *IL-α,* marker of proinflammatory activity ($F_{(1, 13)} = 1.12$, $p>0.30$). Descriptively, *IL-α* levels were higher at ZT12 (RWC,1.05 ± 0.25) compared to ZT0 (AWC,0.90 ± 0.29) with medium effect size (d = 0.55). See figure 9 D.

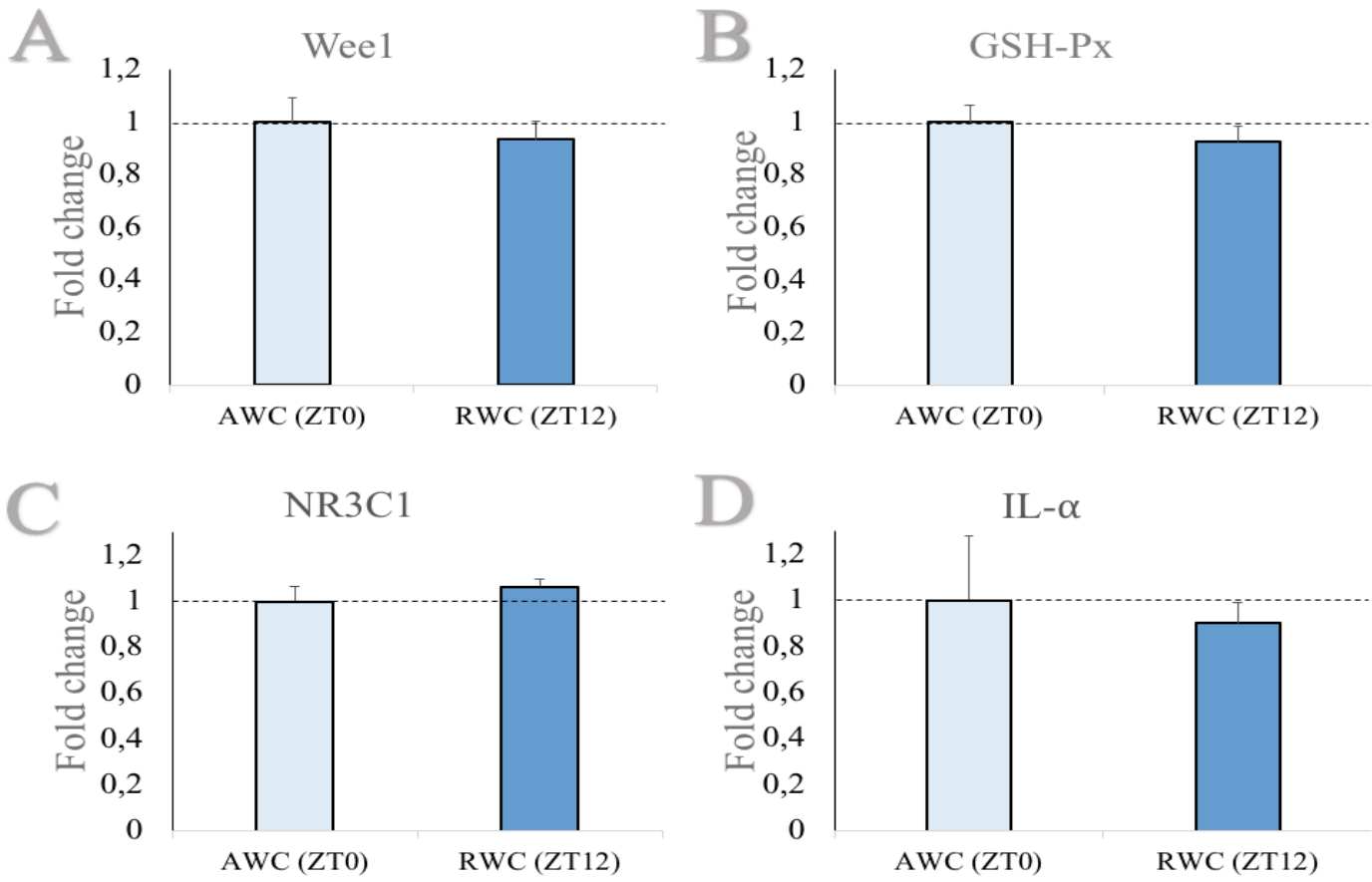


Figure 9. Gene expression of other candidate genes in control animals: rest worker control (RWC, intestinal mucosa sampled at zeitgeber time 12) and active worker control (AWC, intestinal mucosa

sampled at zeitgeber time 0): A) marker of cell cycle checkpoint (*Wee1)*, B) marker oxidative stress *(GSH-Px)*, C) marker of glucocorticoid receptor activity *(NR3C1)* and D) marker of proinflammatory activity (*IL-α)*. Error bars represent standard deviation from the mean.

3.2 Effect of one shift work period

3.2.1 Effect of simulated night shift work.

3.2.1.2 Protein markers in feces. For GSH-PX there was no significant effect of simulated night shift work ($F_{(1, 15)} = 0.71$, $p>0.41$) or simulated night shift work in blue-enriched light ($F_{(1, 13)} = 1.75$, $p>0.20$). Descriptively, the levels of GSH-PX were higher in RW animals (0.80 ± 0.12 U/L) compared to their time matched controls (RWC, ZT12, 0.76 ± 0.06 U/L) with medium effect size (d = 0.42). Furthermore, the levels of GSH-PX were lower in simulated night shift work in blue-enriched light (RWB) animals (0.68 ± 0.14 U/L) compared to time matched control (RWC) (medium effect size, d = -0.74), and compared to simulated night shift work in standard light (large effect size, d = -0.92). See figure 10 A.

For calprotectin there was no significant effect of simulated night shift work ($F_{(1. 15)} = 0.33$, $p>0.57$) or simulated night shift work in blue-enriched light ($F_{(1, 13)} = 0.007$, $p>0.93$). Descriptively, the levels of calprotectin were higher in RW animals (5.93 ± 1.86 ng/g), compared to their time matched controls (RWC, ZT12, 5.26 ± 2.90 ng/g) with small effect size (d = 0.27). Levels of calprotectin in were lower in RWB animals (5.13 ± 2.48 ng/g) compared to their time matched control (RWC) and simulated night shift work in standard light, with small effect size (d = -0.04), and small effect size (-0.36), respectively. See figure 10 C.

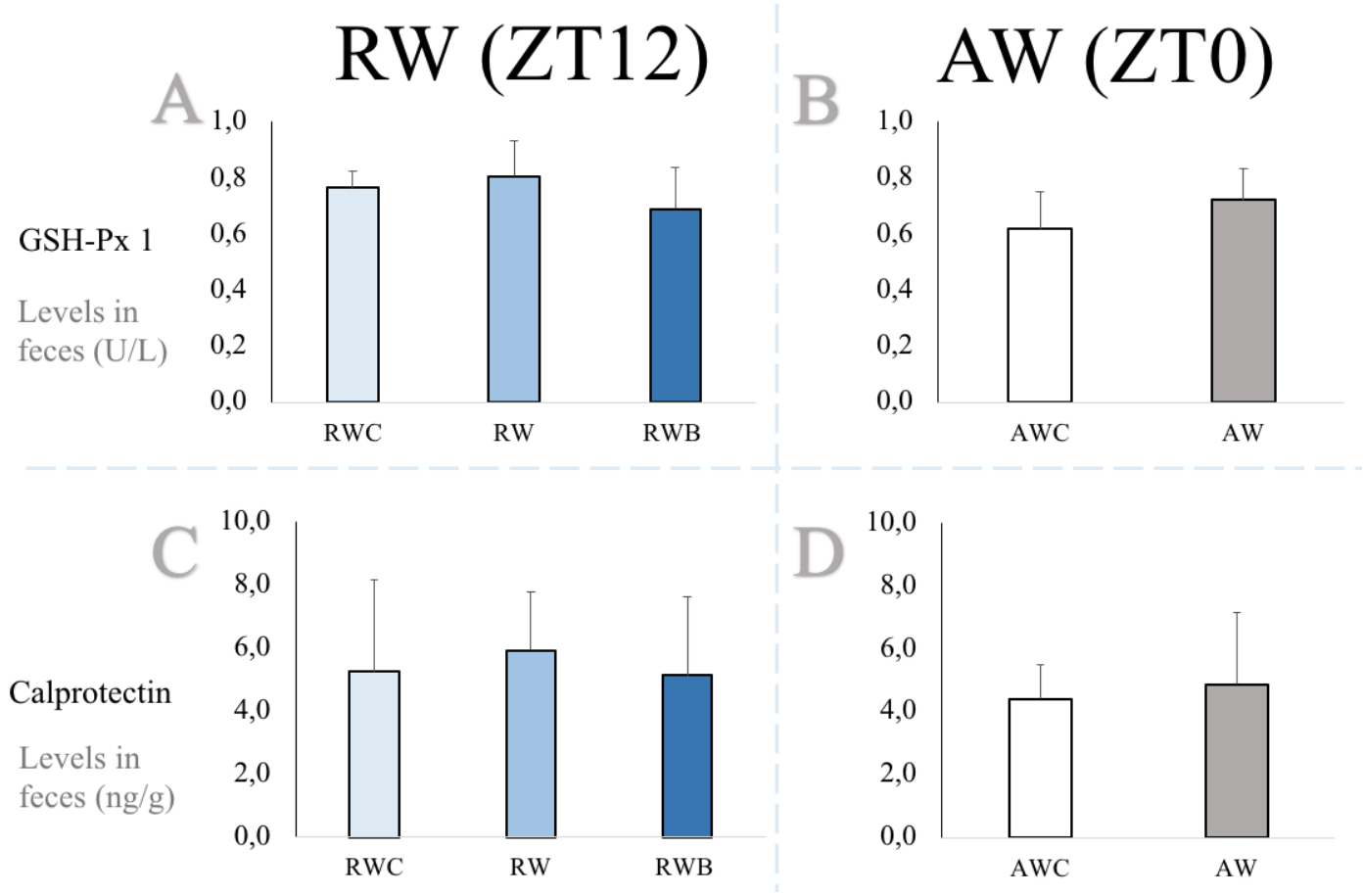


Figure 10. Protein markers in feces of A) oxidative stress (glutathione peroxidase 1, GSH-Px) and C) proinflammatory marker (calprotectin) in rest worker control (RWC, feces sampled at zeitgeber time 12) and experimental groups; rest worker (RW, feces sampled at zeitgeber time 12) and rest worker in blue-enriched light (RWB, feces sampled at zeitgeber time 12); and B) oxidative stress (glutathione peroxidase 1, GSH-Px) and D) proinflammatory activity (calprotectin) in active worker controls (AWC, feces sampled at zeitgeber time 0) and active worker control (AWC, feces sampled at zeitgeber time 0). Error bars represent standard deviation from the mean.

3.2.1.3 mRNA expression in mucosal layer. See figure 11 A and C.

3.2.1.3.1 Clock genes. For *Bmal1* there was a significant effect of simulated night shift work ($F_{(1, 15)} = 4.78$, $p<0.04$). Levels of *Bmal1* were higher in RW animals (1.90 ± 1.04)

compared to their time matched controls (RWC, ZT12, 1.06 ± 0.30). This difference showed a large effect size (d = 1.09).

For *Clock* there was a significant effect of simulated night shift work ($F_{(1, 14)} = 17.63$, $p<0.001$). Levels of *Clock* were higher in RW animals (1.46 ± 0.30) compared to their time matched controls (RWC, ZT12, 0.97 ± .0.04). This difference showed a large effect size (d = 2.28).

For *Per1* there was no significant effect of simulated night shift work ($F_{(1, 15)} = 0.45$, $p>0.51$). Descriptively, the levels of *Per1* were lower in RW animals (0.87 ± 0.39) compared to their time matched controls (RWC, ZT12, 0.99 ± 0.27), with small effect size (d = -0.35).

For *Per2* there was a significant effect of simulated night shift work ($F_{(1, 15)} = 22.53$, $p<0.001$). Levels of *Per2* were higher in RW animals (2.66 ± 0.94) compared to their time matched controls (RWC, ZT12, 1.02 ± 0.25). This difference showed a large effect size (d = 2.38).

Simulated night shift work also had an effect in *RevErb-α* ($F_{(1, 14)} = 22.36$, $p<0.001$). Levels of *RevErb-α* were lower in RW animals (0.44 ± 0.26) compared to their time matched controls (RWC, ZT12, 0.96 ± 0.15). This difference showed a large effect size (d = -2.44). See figure 11 A.

3.2.1.3.2 Other candidate genes. For *Wee1* expression there was a significant effect of simulated night shift work ($F_{(1, 15)} = 13.73$, $p<0.001$). Levels of *Wee1* were higher in RW animals (1.87 ± 0.64) compared to their time matched controls 0.99 ± 0.18). This difference showed a large effect size (d = 1.87).

For *GSH-PX* there was no significant effect of night shift work ($F_{(1, 15)} = 1.69$, $p>0.21$). Descriptively, the levels of *GSH-PX* were lower in RW animals (0.90 ± 0.21)

compared to their time matched controls (RWC, ZT12, 1.03 ± 0.16), with medium effect size (d = -0.69).

For *IL-α* there was no significant effect of night shift work ($F_{(1, 14)} = 0.25$, p>0.62). Descriptively, the levels of *IL-α* were higher in RW animals (1.07 ± 0.45) compared to their time matched controls (0.98 ± 0.24), with medium effect size (d = 0.24).

Also, no effect of night shift work in *NR3C1* expression ($F_{(1, 15)} = 3.39$, p>0.08). Descriptively, the levels of *NR3C1* were higher in RW animals (1.20 ± 0.27) compared to their time matched controls (RWC, ZT12, 1.01 ± 0.09), with large effect size (d = 0.94). See figure 11.

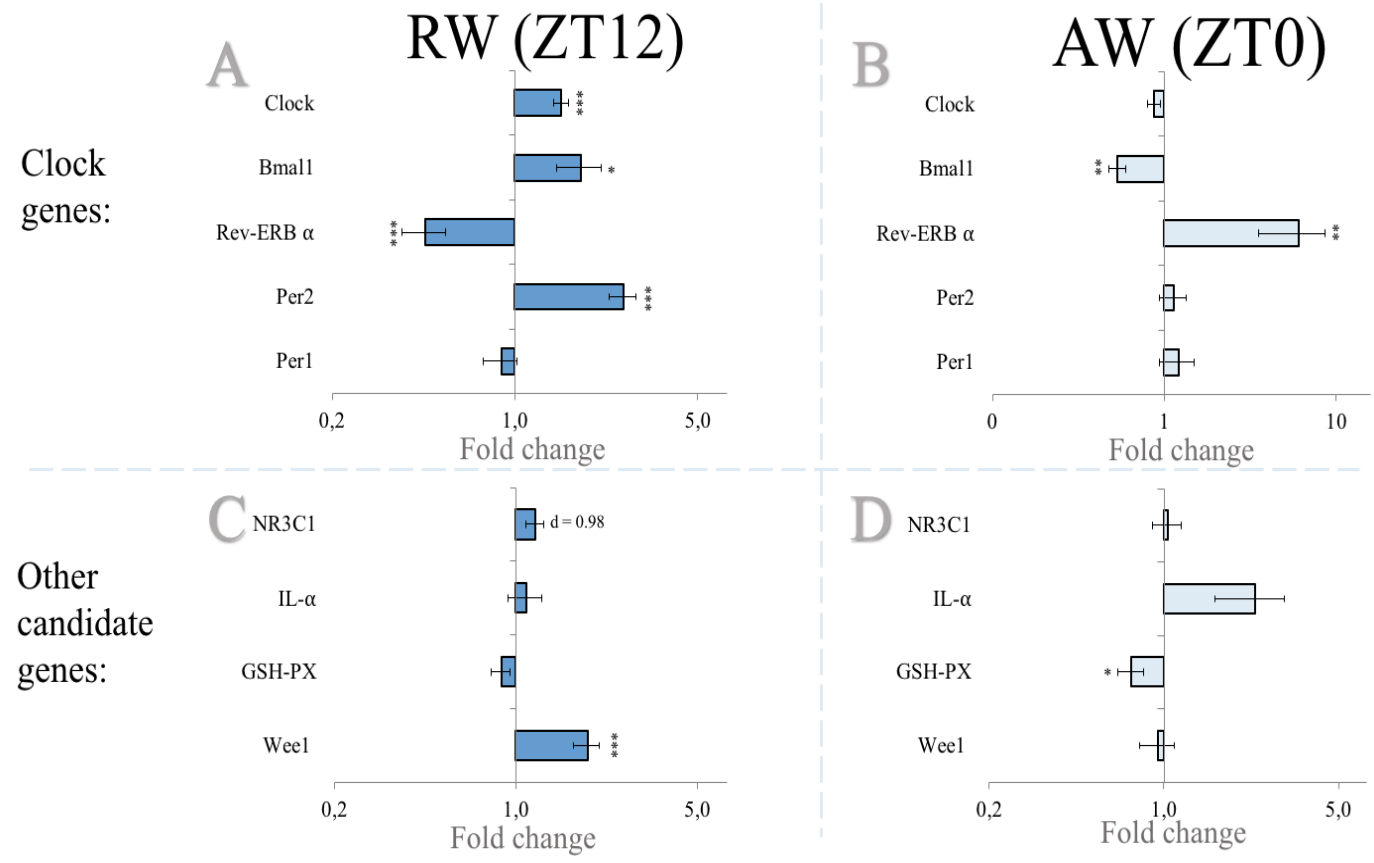


Figure 11. Clock gene expression after one period of shift work in A) simulated night shift work (rest phase workers RW, intestinal mucosa sampled at zeitgeber time 12, ZT12), and B) simulated day shift work (active phase workers AW, intestinal mucosa sampled at zeitgeber time 0, ZT0); Other candidate

gene expression in C) simulated night shift work (rest phase workers, RW, intestinal mucosa sampled at zeitgeber time 12, ZT12), and D) simulated day shift work (active phase workers, AW, intestinal mucosa sampled at zeitgeber time 0, ZT0) relative to control set to fold change 1. Error bars represent standard deviation from the mean; *p<0.05, **p<0.01, ***p<0.001; d= Cohen's d.

3.2.2 Effect of simulated day shift work.

3.2.2.1 Protein markers in feces. For GSH-PX there was no significant effect of simulated day shift work ($F_{(1, 13)} = 2.54$, $p>0.13$). Descriptively, the levels of GSH-PX were higher in AW animals (0.72 ± 0.11 U/L) compared to their time matched controls (AWC, ZT0, 0.61 ± 0.13 U/L) with large effect size (d = 0.83), see figure 10 B.

There was no significant effect of simulated day shift work on levels of calprotectin ($F_{(1, 13)} = 0.24$, $p>0.63$). Descriptively, the levels of calprotectin were higher in AW animals (4.85 ± 2.30 ng/g) compared to their time matched controls (AWC, ZT0, 4.40 ± 1.08 ng/g), with small effect size (d = 0.25), see figure 10 D.

3.2.2.2 mRNA expression in mucosal layer. See figure 11 B and D.

3.2.2.2.1 Clock genes. For *Bmal1* there was a significant effect of simulated day shift work ($F_{(1, 13)} = 36.97$, $p<0.001$). Levels of *Bmal1* were lower in AW animals (0.54 ± 0.15) compared to their time matched controls (AWC, ZT0, 1.00 ± 0.14). This difference showed a large effect size (d = -3.17).

For *Clock* there was no significant effect of simulated day shift work ($F_{(1, 13)} = 2.08$, $p>0.17$). Descriptively, the levels of *Clock* were lower in AW animals (0.88 ± 0.20) compared to their time matched controls (AWC, ZT0, 1.00 ± 0.13), with large effect size (d = -0.71).

For *Per1* there was no significant effect of simulated day shift work ($F_{(1,13)} = 0.63$, $p>0.43$). Descriptively, the levels of *Per1* were higher in AW animals (1.25 ± 0.74) compared to their time matched controls (AWC, ZT0, 1.03 ± 0.28), with small effect size ($d = 0.28$).

For *Per2* there was no significant effect of simulated day shift work ($F_{(1, 13)} = 0.27$, $p>0.60$). Descriptively, the levels of *Per2* were higher in AW animals (1.31 ± 0.54) compared to their time matched controls (AWC, ZT0, 1.15 ± 0.62), with medium effect size ($d = 0.62$).

For *RevErb-α* there was a significant effect of simulated day shift work ($F_{(1, 13)} = 7.98$, $p<0.01$). Levels of *RevErb-α* were higher in AW animals (8.12 ± 6.69) compared to their time matched controls (AWC, ZT0, 1.33 ± 1.22). This difference showed a large effect size ($d = 1.41$.). See figure 11 B.

3.2.2.2.2 Other candidate genes. For *Wee1* there was no significant effect of simulated day shift work ($F_{(1, 12)} = 0.19$, $p>0.66$). Descriptively, the levels of *Wee1* were higher in AW animals (1.00 ± 0.39) compared to their time matched controls (AWC, ZT0, 0.92 ± 0.24), with small effect size ($d = .24$).

For *GSH-PX* there was a significant effect of simulated day shift work ($F_{(1, 13)} = 6.12$, $p<0.02$. Levels of *GSH-PX* were lower in AW animals (0.75 ± 0.23) compared to their time matched controls (AWC, ZT0, 1.01 ± 0.17). This difference showed a large effect size ($d = -1.28$).

For I*L-α* there was no significant effect of simulated day shift work ($F_{(1, 11)} = 3.22 = p>0.10$). Descriptively, the levels of *IL-α* were higher in AW animals (2.09 ± 1.73) compared to their time matched controls (AWC, ZT0, 0.90 ± 0.29), with large effect size ($d = 0.95$).

For *NR3C1* there was no significant effect of simulated day shift work ($F_{(1, 13)} = 0.60$, $p>0.80$). Descriptively, the levels of *NR3C1* were higher in AW animals (1.04 ± 0.35) compared to their time matched controls (AWC, ZT0, 1.01 ± 0.18), with small effect size ($d = 0.10$). See figure 11 D.

4 Discussion

The main aim of this study was to assess the effects of three consecutive days of simulated shift work on gastrointestinal function in rats. In order to do so, first, the time-of-day variation in protein marker of oxidative stress (glutathione peroxidase 1, GSH-Px) and proinflammatory processes (Il-α and calprotectin) in feces was measured. Followed by characterization of clock gene expression (*Clock, Bmal1, Per1, Per2 RevErb-α)* in intestinal mucosa, and expression of other candidate genes involved in proinflammatory processes (*Il-α*); glucocorticoid receptor activity (*NR3C1*); oxidative stress marker (glutathione peroxidase, *GSH-Px*) and cell cycle checkpoint (*Wee1*) measured in the same tissue. Second, the extent to which one simulated shift work period affected these dynamic processes was investigated, in both feces and intestinal mucosa. The present study is the first to look at changes in the gastrointestinal tract following simulated shift work. In the first part of the discussion I will discuss time-of-day variance in protein markers in relation to other findings in different tissues and how they might be translated to our findings in fecal samples. Then I will discuss time-of-day variance in expression of clock genes in relation to other studies findings in the same tissue. In the second part of the discussion I will discuss how one period of shift work affected the processes described above in relation to previous studies on circadian disruption and clock gene function in the colon, and in relation to the association between shift work and negative health outcome related to the function of the gastrointestinal tract.

4.1 Time-of-day variation

4.1.1 Protein markers in feces. There was a time of day dependent variation in oxidative stress marker (glutathione peroxidase) in feces, more specifically levels were higher in transition to active phase at ZT12, compared to transition to inactive phase at ZT0. There are no other studies on this marker in feces, but there some in other tissues. As mentioned initially, glutathione peroxidase levels in plasma and tissue are lower in the inactive phase compared to the active phase both in humans and rats (Cao et al., 2015; Singh et al., 2005). Our results showed an increase in oxidative stress marker in feces at ZT12, which is at the end of the inactive phase. This is the opposite of what I hypothesized to find. In other tissues one might expect that the levels would be lower at ZT12, but since we used feces which is more of an indirect measurement than a direct one, the results are harder to interpret. Since all feces pellets were collected from the colon each sample does not represent one timepoint, but more likely an extended period, possibly of a couple of hours. Previous studies report different total transit times from stomach to colon in rats, generally ranging between 12h to 18h (Dalziel et al., 2016; Varga, 1976). This difference seems to be an effect of age; with longer transit time in older rats, as well as amount of food, where semi-solid versus solid food has a shorter transit time. Thus, this might explain our findings as there is a possibility that it takes time before glutathione peroxidase is transferred from tissue and blood and disposed into the intestinal lumen, and with transit time taken in to account it is reasonable to assume that our measured higher levels at ZT12, compared to ZT0 – in fact represents an accumulation of glutathione levels during the active phase, and not from the inactive phase. Since neither transit time or a delay in secretion was considered in the hypothesis, the results are not in direct opposition if the interpretation is correct. To compare with

a different protein marker in feces: Cinque et al. (2018) observed a time-delay between blood and fecal hormonal levels of corticosterone of 12-18h. Whether this is the case for circadian variation of glutathione peroxidase in feces, remains to determined, as multiple feces sampling at different time points is needed.

Calprotectin levels in feces did not show a time of day variation, which was not surprising given the fact that no other studies have reported one, and since calprotectin is a protein found in neutrophils and used as a marker of inflammation, which will translocate to the outside of the intestinal wall during damage (Jahnsen, Røseth, & Aadlan, 2009). Thus, the results are in accordance with the hypothesis. However, we did observe large individual differences. As there were missing values for interleukin-α no statistical analyses could be performed on this marker. IL-α is constantly present in epithelial cells of the entire gastrointestinal tract (Garlanda, Dinarello, & Mantovani, 2013), and is released in membrane fragments from necrotic cells to initiate inflammation in response to tissue damage (Dinarello, Simon, & Van Der Meer, 2012). Thus, one possible explanation for our non-detections in fecal samples might be that the IL-α containing fragments had not been released from the intestinal wall to feces, and therefore not present in our samples. Another option is that the feces samples did contain IL-α and our homogenization technique was not adequate to free the molecule of from the cell membrane. We used the same procedure for homogenization as was recommended for glutathione peroxidase, since the interleukin kit did not have a recommendation for how to treat fecal samples. The process did not involve steps to ensure a break in the cell membrane which is recommended when extracting nucleic acid or proteins that are located inside the cell (Shehadul Islam, Aryasomayajula, & Selvaganapathy, 2017). Our analysis was preliminary as no other studies have reported to look at IL-α in feces, and given that we only got detectable values for a few rats, each

in different groups, this implicates that the method for the molecular analysis was not appropriate.

4.1.2 Gene expression in the intestinal mucosa of colon. Clock gene expression, measured by differences in mRNA transcription (fold change) showed a significant time-of-day variation for all clock genes with the exception of *Per2*. *Clock* and *Bmal1* which form the positive loop in the primary feedback loop were downregulated and genes forming the negative loop *Per1* and *RevErb-α* were upregulated, after the inactive phase at ZT12. These findings are consistent with the hypothesis and with the literature on timing of expression of clock genes in the rat mucosa in colon (Polidarová et al., 2009; Sládek et al., 2007); see figure 2. Furthermore, Polidarová et al. (2009) also found a relative peak expression of *Wee1* at ZT0 and ZT12, which can be interpreted as consistent with our results as there was no difference between those time points.

For the other candidate genes, mRNA transcription did not show any time-of-day variance, including oxidative stress marker glutathione peroxidase which based on the literature (Baydas et al., 2002) and our feces analysis was expected to have a time-of-day variance. Since we only have two time points measured, we might therefore meet two time points which are relatively similar, compared to what the whole picture might look like. Time-of-day variation was neither found in mRNA levels for the genes encoding *IL-α* or *NR3C1* which are the proinflammatory marker and glucocorticoid receptor, respectively. There were large individual differences in mRNA levels of the proinflammatory marker. For future research measurement at multiple time points are needed throughout 24 h to is needed to get an accurate description of circadian cycle of clock genes, but also of other genes that might be under the circadian-clock influence.

4.2 Effect of one shift work period

In our experimental protocol, rest phase workers (RW) and active phase workers (AW) were euthanized at different zeitgeber time points and therefore compared to time-matched controls only; therefore, AW servers as an extra control group for forced activity.

4.2.1 Protein markers in feces after simulated shift work. Our results gave no significant differences in oxidative stress (glutathione peroxidase) or the proinflammatory marker (calprotectin) in feces in either rest workers or active workers compared to their time-matched controls. Only descriptively, glutathione peroxidase was increased as an effect of work, as both rest workers (RW) and active workers (AW) showed an increase with a medium and a large effect size, respectively. This seems to be consistent with previous research indicating that oxidative stress has the potential to increase after physical exercise (Fisher-Wellman & Bloomer, 2009). Together, with our time-of-day finding of higher levels at ZT12 in feces, this would suggest that rest workers have an increase in glutathione peroxidase levels after simulated night shift work. However, rest workers worked with lights on, which might reduce glutathione peroxidase levels, as a previous study found that constant light reduces glutathione peroxidase activity in rat brain, liver and kidney (Baydaş, Erçel, Canatan, Dönder, & Akyol, 2001). In a follow up study, the authors suggest a reduction of the hormone melatonin to be responsible for this effect as it reaches peak levels earlier than glutathione peroxidase, and animals with inhibited melatonin secretion had a reduced peak levels of glutathione peroxidase, which was restored when they got administration of melatonin (Baydas et al., 2002). As light suppresses melatonin secretion in both rats and humans, a possible explanation why we did not find a difference between rest workers and their time-matched controls is that light by suppressing melatonin secretion countered an expected increase in glutathione

peroxidase in rest workers. Furthermore, being active in the rest phase is considered as a stressor, and stress has been shown to increase transit time in colon (Williams, Villar, Peterson, & Burks, 1988), which might shorten the time of accumulation of glutathione peroxidase in feces before being sampled. One interesting finding although not significant is that rest work in blue-enriched light decreased glutathione peroxidase levels compared to time-matched controls and rest phase workers in standard lightning showing a large effect size for both. Again, possibly due to a greater suppression of melatonin? The hypothesis of increased oxidative stress following simulated night shift work, as measured by glutathione peroxidase levels, was not confirmed. Whether melatonin is involved or had an impact on the results our study cannot answer, since we did not measure melatonin levels. Future research should consider including measurement of melatonin levels both in plasma and tissue.

No significant differences were found between calprotectin levels in rest phase workers or active workers relative to their time-matched controls (with large individually differences and small effect sizes). This indicates that three consecutive days of shift work did not cause an upregulation of pro-inflammatory markers in the intestine. Other studies on chronic circadian disruption that reported upregulation of inflammatory markers have used more extreme disruption of the circadian rhythm, such as rapid changes in light/dark cycle or constant light for an extended period of two weeks to a month (Polidarová et al., 2017; Preuss et al., 2008), while our experiment looked at acute effects of simulated shift work (three days).

4.2.2 Gene expression in the intestinal mucosa of colon after simulated shift work. After the animals had been exposed to three consecutive days of rest phase work (RW) we observed transcriptional alterations in several clock genes, with multiple genes changing

transcriptional direction. Regarding the positive loop of the clock machinery, transcription of *Clock* and *Bmal1* was upregulated compared to their time-matched controls. Regarding the negative loop, transcription of *Per2* was upregulated nearly three-fold, whereas *RevErb-α* transcription was downregulated. Transcriptional levels of *Per1* were downregulated although not significant and with a small effect size. This change in direction (up- or downregulation) in different clock genes is suggestive of a dysregulation, but as we only have measured one specific time point for both RW and AW, we cannot conclude if their rhythm is out of synchrony and if a change in phase had occurred. Again, future studies need multiple measurements throughout 24h to verify our finding. In active phase workers (AW) transcriptional alterations were observed for only two clock genes; levels of *RevErb-α* were upregulated eight-fold, but with large individually differences, while a less pronounced downregulation of *Bmal1* was present in AW compared to their time matched controls. This might be due to the fact that *RevErb-α* regulates expression of metabolic genes (Cho et al., 2012) and our animals were under forced activity and with free access to food and water, which can cause transcription activation of *RevErb-α,* thus explaining the upregulation in AW. In addition, since *RevErb-α* was highly upregulated it might explain why *Bmal1* was downregulated since *RevErb*-α acts as a repressor on its transcription (Haus & Smolensky, 2013).

Together, these data demonstrate that clock gene expression after three days of simulated shift work was more affected in RW than in AW, thus in accord the hypothesis. Correspondingly, the same experimental protocol in an earlier study from our laboratory induced alteration in clock gene expression in the rat liver of RW (unpublished data). In a study from Polidarová et al. (2011) constant light and access to food *ad libitum* was used to eliminate entrainment signals from SCN and food, which

resulted in loss of rhythmic expression of *Per1, Per2, Bmal1* and *Wee1* in colon. By introducing a restricted feeding schedule, all clock genes became rhythmic with the exception of *Per2* and *Wee1*. In contrast, all clock genes became rhythmically expressed after restricted feeding in the duodenum and liver. The authors suggest that this implies that in the colon, SCN competes with food as an entraining cue. This might explain why clock genes in our model were out of synchrony; in our model all animals get access to food *ad libitum* during the experiment, and Marti et al. (2016) found that RW shift their food intake to be predominantly in their rest phase after day 3. Based on this finding we can assume that our rest workers get conflicting entrainment cues, as we can assume that light ON entrains the clock genes in SCN and food intake entrains the peripheral clocks in colon. In addition, if we rely on Polidarova´s group findings, food is not enough to synchronize all clock genes in colon, causing a malfunction in synchronization of rhythmicity.

In line with these findings, in RW there was a transcriptional alternation in cell cycle checkpoint gene *Wee1* which was upregulated compared to time-matched controls. There was no difference in transcription of this gene in AW, indicating that the upregulation in RW was an effect of simulated night shift work and not forced activity in general. *Wee1* is a gatekeeper of the cell cycle, as it can arrest the cell for DNA repair before it goes into mitosis (cell division), thus hindering replication of damaged DNA. Dysregulation of this gene has been implicated in different cancer types (Matheson, Backos, & Reigan, 2016). High expression has been reported in several cancer types and associated with tumor progression and poor rates of survival, including colorectal cancer (Egeland et al., 2016; Ge et al., 2017). Our finding of increased expression of *wee1* might therefore be related to the increased risk of colorectal cancer seen among

night shift workers (X. Wang et al., 2015), making *Wee1* dysregulation a possible mechanism.

Per2 in RW was upregulated compared to time-matched controls. Clock gene *Per2* has been claimed to play a part in motility regulation by Hoogerwerf et al. (2009). If *Per2* is involved in motility and its rhythm is disturbed by night shift work, this might be one possible explanation of the gastrointestinal complaints of abdominal pain, diarrhea or constipation reported in shift workers (Knutsson & Bøggild, 2010) since changes in motility may cause discomfort or other problems with transition. Motility changes are also considered as one of the causing agents of irritable bowel syndrome, and as Nojkov et al. (2010) showed, symptoms of irritable bowel syndrome are prevalent among shift workers.

RevErb-α on the other hand, which normally follows *Per2* rhythm, was downregulated in RW. This seems to be in line with findings by S. Wang et al. (2018) where jet-lag led to a downregulation of RevErb-α in the colon of mice. Furthermore, they also found that mice lacking this gene developed a more severe DSS-induced colitis compared to wild-type mice suggesting a central role for *RevErb-α* in the association between circadian disruption and worsening of DSS-induced colitis via proinflammatory processes. As an explanation they found *Reverb-α* to directly suppress transcription of a clock-controlled gene complex (*Nlrp3*) that promotes proinflammatory interleukin expression (IL-β and IL-18) (S. Wang et al., 2018). *Reverb-α* can also repress NF-kB pathway, which is a family of transcriptional factors that regulates expression of a number of genes involved in proinflammatory processes and apoptosis (Sato et al., 2014). Moreover, a number of autoimmune diseases are associated with NF-kB activation including irritable bowel disease and ulcerative colitis (Lawrence, 2009). Taken together, this would imply that *RevErb-α* functions as a gatekeeper in the interconnection between

circadian clock and the immune system. To further support this assumption, administering a protein agonist of *Reverb-α* to mice before giving them the DSS treatment led to a significant reduction in symptoms of colitis compared with mice only treated with DSS (S. Wang et al., 2018).Whether *RevErb-α* is involved in proinflammatory processes in our model should be investigated in the future.

Alteration in glutathione peroxidase as marker of oxidative stress was only found in AW, showing a downregulation of mRNA levels. One of the hypotheses of this thesis was that there would be an increase in oxidative stress in RW, which we did not find. In the literature, high levels of glutathione peroxidase are assumed to be a sign of higher degree of oxidative stress as reactive oxygen species accumulation will make the body produce more of the antioxidant enzyme to counter the imbalance. However, it might also be the opposite – in the absence of reactive oxidative species there is no oxidative stress to be reduced and glutathione peroxidase levels accumulate (Lubos et al., 2011). It is hard to conclude why there was a reduction in expression of glutathione peroxidase levels in AW and no difference in RW after one period of shift work, since we did not measure any marker of reactive oxygen species – which could have given more information about the state of oxidative stress.

In terms of inflammation as measured by interleukin-α there was no change in this marker in either RW or AW compared to their-time matched controls. However, a borderline significant upregulation in gene encoding for glucocorticoid receptor activity (*NR3C1*) was found in RW, showing a large effect size ($p>0.08$, $d=0.94$). Given the large effect size it is reasonable to assume this upregulation was not coincidental. An increase of glucocorticoid receptor activity is indicative of higher stress hormone activity, which could mediate proinflammatory activity throughout other markers not investigated in this experiment.

4.3 Evaluation of the present experiment and methods

4.3.1 Strengths and limitations. One strength of this experiment is that it uses an already established model of shift work that has been validated as a model for human shift work (Grønli et al., 2017; Marti et al., 2016; Marti et al., 2017). Furthermore, it also includes active phase workers which allow us to control for the forced activity protocol.

Limitations in the current experiment includes limited sample size, which may have reduced statistical power and increased risk of type II errors (false negative). In addition, the small sample size made it possible to only asses one time point, making the results hard to interpret, as there is a possibility that the changes in the mRNA expression after simulated night shift work are small transcriptional fluctuations and not more stable changes. Furthermore, we only have mRNA data and not protein data. A lot of posttranscriptional changes can occur before the end product of proteins are made (Day & Tuite, 1998). If more animals were available it would not only increase the strength but also give us a more complete picture of how working in the rest phase affects different markers and clock gene expression. Other limitations are related to the methods for collection of samples and analysis method for both tissue and feces samples. Sampling of feces is a useful non-invasive method to asses' biomarkers, which offers strengths and limitations; the main limitation is that it is rarely used in the literature, as blood sampling remains dominant. There are a few studies which have used feces to measure corticosterone in rats, equivalent to cortisol in humans, but this entails a somewhat different procedure than the one we used to measure glutathione peroxidase and calprotectin. Hence, how to best ensure homogenization of feces pellets was a challenge. For future research we propose to use liquid nitrogen to freeze then crush and mix feces pellets thoroughly. An advantage of using feces is that it provides an averaged

measure of a marker related to a time window depending on intestinal transit time; thus, making the marker less affected by short-time small fluctuations that might take place before sampling.

Gene expression analysis with the use of real-time polymerase chain reaction (RT-PCR) offers strength since it is highly sensitive and can detect lower molecular levels than other diagnostic methods. On the other hand, one concern with highly sensitive tests is that even the smallest contamination in the sample can increase risk of type I error (false positive). Our samples are from a complex biological site (intestinal mucosa) and might contain substances that interfere with the many complex molecular reactions upon which the tests rely. This is the first time our research group is using intestinal tissue for RT-PCR, and the literature using the same method and tissue was not very specific in how to proceed throughout harvesting tissue and analysis other than referring to the manufacturer's recommendations. The establishment of the method in our laboratory needs therefore further improvement, as reliable RT-PCR results were only detectable in colon tissue, but not in duodenum. This is curious; one explanation might be that the duodenum contains a lot of digestive enzymes compared to the colon, and is therefore more susceptible to degradation. Another factor might be that our colon samples were diluted in a double amount of lysis buffer enabling easier RNA isolation. For nucleic acid concentration in the different tissue segments after RNA isolation, see appendix.

Furthermore, there are a few enhancements of the method that should be incorporated in the future. One, is removal of the intestine as soon as possible after euthanization – in our experiment other tissues were harvested before intestinal tissue, thus extending the degradation time of RNA. In addition, using a RNAlater stabilization reagent on tissue directly after being collected might reduce degradation. This solution

inactivates RNase (enzyme which destroys RNA) and therefore stabilizes and protects cellular RNA. Moreover, our animals were not fasted before harvesting the tissue which can affect gene expression of transcripts related to metabolism, also possibly affecting intestinal bacteria. These aspects should be taken into consideration for future experiments.

4.3.2 Future perspectives. The present rat model of simulated shift work has characterized the effects of time-of-day variation in gene expression in intestinal tissue and protein marker in feces, and how these dynamic processes are affected by either rest phase work or active phase work at the end of work day 3 only. Future research should aim at characterizing these effects throughout the entire 3 days of shift work period. In addition, by increasing the periods of shift work it is possible to look at long-term effects after working on a prolonged shift work schedule. Furthermore, data from recovery in between shift work periods would also give valuable information about how long it takes before the changes in gene expression return to normal. Of interest, our results showed a large transcriptional upregulation of *Wee1* after three consecutive days of simulated night shift work, and future studies should be conducted to determine the daily gene expression profile of *Wee1* in rest phase workers, as it seems to be lacking in the literature in colon tissue. Furthermore, with the use of the animal model of shift work, sampling at multiple time points throughout the entire 24-h circadian cycle should be incorporated to determine how exactly the rhythm of the cell cycle gene is affected and how long it takes before the rhythm returns to normal (recovery period).

4.4 Conclusion

The results of the present study show that one shift work period (three consecutive shifts) of rest phase work is enough to induce changes in clock gene expression in the colon. However, whether this is reflecting a desynchronization or a phase shift is not possible to conclude from our data set with a single time point. Normally clock genes in the positive loop would be downregulated at the end of the rest phase, while clock genes in the negative loop would be upregulated. In our sample of rest phase workers, the positive loop genes were upregulated, while genes in the negative loop were in disarray as they were both upregulated and downregulated. In addition, as the levels of the clock-controlled gene *wee1* (cell cycle checkpoint*)* were only affected in rest phase workers, this could imply a possible mechanism behind the association between increased risk of colorectal cancer seen among night shift workers, which should be investigated. This study combined with previous studies has elucidated possible factors that can contribute to negative health effects of the gastrointestinal tract in shift workers and should encourage future studies to be carried out based on some of the findings.

5 References

Ahluwalia, B., Magnusson, M. K., & Öhman, L. (2017). Mucosal immune system of the gastrointestinal tract: maintaining balance between the good and the bad. *Scandinavian journal of gastroenterology, 52*(11), 1185-1193. doi:10.1080/00365521.2017.1349173

Åkerstedt, T. (2003). Shift work and disturbed sleep/wakefulness. *Occupational Medicine, 53*(2), 89-94. doi:10.1093/occmed/kqg046

Aschoff, J. (1965). Circadian rhythms in man. *Science, 148*(3676), 1427-1432. doi:10.1126/science.148.3676.1427

Bandyopadhyay, U., Das, D., & Banerjee, R. K. (1999). Reactive oxygen species: oxidative damage and pathogenesis. *Current Science-Bangalore-, 77*(5), 658-666. Retrieved from: www.jstor.org/stable/24102839.

Barnes, P. J., Adcock, I., Spedding, M., & Vanhoutte, P. M. (1993). Anti-inflammatory actions of steroids: molecular mechanisms. *Trends in pharmacological sciences, 14*(12), 436-441. doi:10.1016/0165-6147(93)90184-L

Baydaş, G., Erçel, E., Canatan, H., Dönder, E., & Akyol, A. (2001). Effect of melatonin on oxidative status of rat brain, liver and kidney tissues under constant light exposure. *Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease, 19*(1), 37-41. doi:10.1002/cbf.897

Baydas, G., Gursu, M. F., Yilmaz, S., Canpolat, S., Yasar, A., Cikim, G., & Canatan, H. (2002). Daily rhythm of glutathione peroxidase activity, lipid peroxidation and glutathione levels in tissues of pinealectomized rats. *Neuroscience letters, 323*(3), 195-198. doi:10.1016/S0304-3940(02)00144-1

Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature Reviews Genetics, 6*(7), 544-556. doi:10.1038/nrg1633

Berson, D. M. (2003). Strange vision: ganglion cells as circadian photoreceptors. *TRENDS in Neurosciences, 26*(6), 314-320. doi:10.1016/S0166-2236(03)00130-9

Bharucha, A. E., & Brookes, S. J. (2018). Neurophysiologic mechanisms of human large intestinal motility *Physiology of the Gastrointestinal Tract (Sixth Edition)* (pp. 517-564): Elsevier.

Binder, H. J. (2006). Causes of chronic diarrhea. *New England Journal of Medicine, 355*(3), 236. Retrieved from: https://www.nejm.org/doi/pdf/10.1056/NEJMp068124

Bjarnason, G. A., & Jordan, R. (2002). Rhythms in human gastrointestinal mucosa and skin. *Chronobiology international, 19*(1), 129-140. doi:10.1081/CBI-120002595

Cao, H., Qin, F., Liu, X., Wang, J., Cao, Y., Tong, J., & Zhao, H. (2015). Circadian rhythmicity of antioxidant markers in rats exposed to 1.8 GHz radiofrequency fields. *International journal of environmental research and public health, 12*(2), 2071-2087. doi:10.3390/ijerph120202071

Cappuccio, F. P., Taggart, F. M., Kandala, N.-B., Currie, A., Peile, E., Stranges, S., & Miller, M. A. (2008). Meta-analysis of short sleep duration and obesity in children and adults. *Sleep, 31*(5), 619-626. doi:10.1093/sleep/31.5.619

Cedernaes, J., Schönke, M., Westholm, J. O., Mi, J., Chibalin, A., Voisin, S., . . . Dickson, S. L. (2018). Acute sleep loss results in tissue-specific alterations in genome-wide DNA methylation state and metabolic fuel utilization in humans. *Science advances, 4*(8), 1-14. doi:10.1126/sciadv.aar8590

Cho, H., Zhao, X., Hatori, M., Ruth, T. Y., Barish, G. D., Lam, M. T., . . . Glass, C. K. (2012). Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. *Nature, 485*, 123-127. doi:10.1038/nature11048

Cinque, C., Zinni, M., Zuena, A. R., Giuli, C., Alemà, S. G., Catalani, A., . . . Cozzolino, R. (2018). Faecal corticosterone metabolite assessment in socially housed male and female Wistar rats. *Endocrine connections, 7*(2), 250-257. doi:10.1530/EC-17-0338

Costa, G. (2003). Shift work and occupational medicine: an overview. *Occupational Medicine, 53*(2), 83-88. doi:10.1093/occmed/kqg045

Costa, G. (2010). Shift Work and Health: Current Problems and Preventive Actions. *Safety and Health at Work, 1*(2), 112-123. doi:10.5491/SHAW.2010.1.2.112

Czeisler, C. A., Duffy, J. F., Shanahan, T. L., Brown, E. N., Mitchell, J. F., Rimmer, D. W., . . . Emens, J. S. (1999). Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science, 284*(5423), 2177-2181. doi:10.1126/science.284.5423.2177

Dalziel, J., Young, W., Bercik, P., Spencer, N., Ryan, L., Dunstan, K., . . . Roy, N. (2016). Tracking gastrointestinal transit of solids in aged rats as pharmacological models of chronic dysmotility. *Neurogastroenterology & Motility, 28*(8), 1241-1251. doi:10.1111/nmo.12824

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & development, 14*(23), 2950-2961. doi:10.1101/gad.183500

Day, D., & Tuite, M. F. (1998). Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. *The Journal of endocrinology, 157*(3), 361-371. doi:10.1677/joe.0.1570361

de Almeida, C. M. O., & Malheiro, A. (2016). Sleep, immunity and shift workers: a review. *Sleep science, 9*(3), 164-168. doi:10.1016/j.slsci.2016.10.007

Dinarello, C. A., Simon, A., & Van Der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature reviews Drug discovery, 11*(8), 633-652. doi:doi.org/10.1038/nrd3800

Do, M. T. H., & Yau, K.-W. (2010). Intrinsically photosensitive retinal ganglion cells. *Physiological reviews, 90*(4), 1547-1581. doi:10.1152/physrev.00013.2010

Egeland, E. V., Flatmark, K., Nesland, J. M., Flørenes, V. A., Mælandsmo, G. M., & Boye, K. (2016). Expression and clinical significance of Wee1 in colorectal cancer. *Tumor Biology, 37*(9), 12133-12140. doi:10.1007/s13277-016-5081-3

Fisher-Wellman, K., & Bloomer, R. J. (2009). Acute exercise and oxidative stress: a 30 year history. *Dynamic medicine, 8*(1), 1-25. doi:10.1186/1476-5918-8-1

Folkard, S. (2008). Do permanent night workers show circadian adjustment? A review based on the endogenous melatonin rhythm. *Chronobiology international, 25*(2-3), 215-224. doi:10.1080/07420520802106835

Froy, O., & Chapnik, N. (2007). Circadian oscillation of innate immunity components in mouse small intestine. *Molecular immunology, 44*(8), 1954-1960. doi:10.1016/j.molimm.2006.09.026

Garlanda, C., Dinarello, C. A., & Mantovani, A. (2013). The interleukin-1 family: back to the future. *Immunity, 39*(6), 1003-1018. doi:10.1016/j.immuni.2013.11.010

Garrett, W. S., Gordon, J. I., & Glimcher, L. H. (2010). Homeostasis and inflammation in the intestine. *Cell, 140*(6), 859-870. doi:10.1016/j.cell.2010.01.023

Ge, X. C., Wu, F., Li, W. T., Zhu, X. J., Liu, J. W., & Wang, B. L. (2017). Upregulation of WEE1 is a potential prognostic biomarker for patients with colorectal cancer. *Oncology letters, 13*(6), 4341-4348. doi:10.3892/ol.2017.5984

Goichot, B., Weibel, L., Chapotot, F., Gronfier, C., Piquard, F., & Brandenberger, G. (1998). Effect of the shift of the sleep-wake cycle on three robust endocrine markers of the circadian clock. *American Journal of Physiology-Endocrinology And Metabolism, 275*(2), E243-E248. doi:10.1152/ajpendo.1998.275.2.E243

Grønli, J., Meerlo, P., Pedersen, T. T., Pallesen, S., Skrede, S., Marti, A. R., . . . Mrdalj, J. (2017). A Rodent Model of Night-Shift Work Induces Short-Term and Enduring Sleep and Electroencephalographic Disturbances. *Journal of Biological Rhythms, 32*(1), 48-63. doi:10.1177/0748730416675460

Haus, E. L., & Smolensky, M. H. (2013). Shift work and cancer risk: potential mechanistic roles of circadian disruption, light at night, and sleep deprivation. *Sleep medicine reviews, 17*(4), 273-284. doi:10.1016/j.smrv.2012.08.003

Hoogerwerf, W. A., Hellmich, H. L., Cornélissen, G., Halberg, F., Shahinian, V. B., Bostwick, J., . . . Cassone, V. M. (2007). Clock gene expression in the murine gastrointestinal tract: endogenous rhythmicity and effects of a feeding regimen. *Gastroenterology, 133*(4), 1250-1260. doi:10.1053/j.gastro.2007.07.009

Hoogerwerf, W. A., Shahinian, V. B., Cornélissen, G., Halberg, F., Bostwick, J., Timm, J., . . . Cassone, V. M. (2009). Rhythmic changes in colonic motility are regulated by period genes. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 298*(2), G143-G150. doi:10.1152/ajpgi.00402.2009.

Hoogerwerf, W. A., Sinha, M., Conesa, A., Luxon, B. A., Shahinian, V. B., Cornélissen, G., . . . Cassone, V. M. (2008). Transcriptional profiling of mRNA expression in the mouse distal colon. *Gastroenterology, 135*(6), 2019-2029. doi:10.1053/j.gastro.2008.08.048

Iwashina, I., Mochizuki, K., Inamochi, Y., & Goda, T. (2011). Clock genes regulate the feeding schedule-dependent diurnal rhythm changes in hexose transporter gene expressions

through the binding of BMAL1 to the promoter/enhancer and transcribed regions. *The Journal of nutritional biochemistry, 22*(4), 334-343. doi:10.1016/j.jnutbio.2010.02.012

Jahnsen, J., Røseth, A. G., & Aadlan, E. (2009). Måling av kalprotektin i feces. *Tidsskriftet den Norske Legeforening, 8*(129), 743-745. doi:10.4045/tidsskr.08.0010

Kalsbeek, A., la Fleur, S., & Fliers, E. (2014). Circadian control of glucose metabolism. *Molecular metabolism, 3*(4), 372-383. doi:10.1016/j.molmet.2014.03.002

Karaus, M., & Wienbeck, M. (1991). Colonic motility in humans—a growing understanding. *Bailliere's clinical gastroenterology, 5*(2), 453-478. doi:10.1016/0950-3528(91)90037-2

Karlsson, B., Knutsson, A., & Lindahl, B. (2001). Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. *Occupational and environmental medicine, 58*(11), 747-752. doi:10.1136/oem.58.11.747

Knutsson, A. (2003). Health disorders of shift workers. *Occupational Medicine, 53*(2), 103-108. doi:10.1093/occmed/kqg048

Knutsson, A., & Bøggild, H. (2010). Gastrointestinal disorders among shift workers. *Scandinavian Journal of Work, Environment & Health, 36*(2), 85-95. doi:10.5271/sjweh.2897

Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Human molecular genetics, 15*(2), R271-R277. doi:10.1093/hmg/ddl207

Kolker, D., & Turek, F. W. (1999). The search for circadian clock and sleep genes. *Journal of Psychopharmacology, 13*(4), S5-S9. doi:10.1177/026988119901304S02

Kowalska, E., & Brown, S. (2007). *Peripheral clocks: keeping up with the master clock.* Paper presented at the Cold Spring Harbor Symposia on Quantitative Biology.

Lac, G., & Chamoux, A. (2004). Biological and psychological responses to two rapid shiftwork schedules. *Ergonomics, 47*(12), 1339-1349. doi:10.1080/00140130410001724237

Lawrence, T. (2009). The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor perspectives in biology, 1*(6), 1-10. doi:10.1101/cshperspect.a001651

Louis, P., Scott, K. P., Duncan, S. H., & Flint, H. J. (2007). Understanding the effects of diet on bacterial metabolism in the large intestine. *Journal of applied microbiology, 102*(5), 1197-1208. doi:10.1111/j.1365-2672.2007.03322.x

Lubos, E., Loscalzo, J., & Handy, D. E. (2011). Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxidants & redox signaling, 15*(7), 1957-1997. doi:10.1089/ars.2010.3586

Macpherson, A. J., & Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nature reviews immunology, 4*(6), 478. doi:10.1038/nri1373

Marti, A. R., Meerlo, P., Grønli, J., Vanhasselt, S., Mrdalj, J., Pallesen, S., . . . Skrede, S. (2016). Shift in Food Intake and Changes in Metabolic Regulation and Gene Expression during Simulated Night-Shift Work: A Rat Model. *Nutrients, 8*(11), 712. doi:10.3390/nu8110712

Marti, A. R., Patil, S., Mrdalj, J., Meerlo, P., Skrede, S., Pallesen, S., . . . Grønli, J. (2017). No escaping the rat race: simulated night shift work alters the time-of-day variation in BMAL1 translational activity in the prefrontal cortex. *Frontiers in neural circuits, 11*, 70. doi:10.3389/fncir.2017.00070

Matheson, C. J., Backos, D. S., & Reigan, P. (2016). Targeting WEE1 kinase in cancer. *Trends in pharmacological sciences, 37*(10), 872-881. doi:10.1016/j.tips.2016.06.006

Matsuo, T., Yamaguchi, S., Mitsui, S., Emi, A., Shimoda, F., & Okamura, H. (2003). Control mechanism of the circadian clock for timing of cell division in vivo. *Science, 302*(5643), 255-259. doi:10.1126/science.1086271

McNeil, N. (1984). The contribution of the large intestine to energy supplies in man. *The American journal of clinical nutrition, 39*(2), 338-342. doi:10.1093/ajcn/39.2.338

Meerlo, P., Sgoifo, A., & Suchecki, D. (2008). Restricted and disrupted sleep: effects on autonomic function, neuroendocrine stress systems and stress responsivity. *Sleep medicine reviews, 12*(3), 197-210. doi:10.1016/j.smrv.2007.07.007

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & redox signaling, 20*(7), 1126-1167. doi:10.1089/ars.2012.5149

Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain research, 42*(1), 201-206. doi:10.1016/0006-8993(72)90054-6

Nojkov, B., Rubenstein, J. H., Chey, W. D., & Hoogerwerf, W. A. (2010). The impact of rotating shift work on the prevalence of irritable bowel syndrome in nurses. *The American journal of gastroenterology, 105*(4), 842-847. doi:10.1038/ajg.2010.48.

Oh-oka, K., Kono, H., Ishimaru, K., Miyake, K., Kubota, T., Ogawa, H., . . . Nakao, A. (2014). Expressions of tight junction proteins Occludin and Claudin-1 are under the circadian control in the mouse large intestine: implications in intestinal permeability and susceptibility to colitis. *PloS one, 9*(5), 1-10. doi:10.1371/journal.pone.0098016

Opperhuizen, A.-L., van Kerkhof, L. W., Proper, K. I., Rodenburg, W., & Kalsbeek, A. (2015). Rodent models to study the metabolic effects of shiftwork in humans. *Frontiers in pharmacology, 6*, 1-20. doi:10.3389/fphar.2015.00050

Pan, A., Schernhammer, E. S., Sun, Q., & Hu, F. B. (2011). Rotating night shift work and risk of type 2 diabetes: two prospective cohort studies in women. *PLoS medicine, 8*(12), 1-8. doi:10.1371/journal.pmed.1001141

Pan, X., & Hussain, M. M. (2009). Clock is important for food and circadian regulation of macronutrient absorption in mice. *Journal of lipid research, 50*(9), 1800-1813. doi:10.1194/jlr.M900085-JLR200

Panda, S., Hogenesch, J. B., & Kay, S. A. (2002). Circadian rhythms from flies to human. *Nature, 417*(6886), 329-335. doi:10.1038/417329a

Perry, J. A., & Kornbluth, S. (2007). Cdc25 and Wee1: analogous opposites? *Cell division, 2*(1), 1-12. doi:10.1186/1747-1028-2-12

Pittendrigh, C. S., & Minis, D. H. (1964). The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *The American Naturalist, 98*(902), 261-294. doi:10.1086/282327

Polidarová, L., Houdek, P., & Sumová, A. (2017). Chronic disruptions of circadian sleep regulation induce specific proinflammatory responses in the rat colon. *Chronobiology international, 34*(9), 1273-1287. doi:10.1080/07420528.2017.1361436

Polidarová, L., Sládek, M., Soták, M., Pácha, J., & Sumová, A. (2011). Hepatic, duodenal, and colonic circadian clocks differ in their persistence under conditions of constant light and in their entrainment by restricted feeding. *Chronobiology international, 28*(3), 204-215. doi:10.3109/07420528.2010.548615

Polidarová, L., Soták, M., Sládek, M., Pácha, J., Sumová, A., Polidarová, L., . . . Sumová, A. (2009). Temporal gradient in the clock gene and cell-cycle checkpoint kinase Wee1 expression along the gut. *Chronobiology international, 26*(4), 607-620. doi:10.1080/07420520902924889

Preuss, F., Tang, Y., Laposky, A. D., Arble, D., Keshavarzian, A., & Turek, F. W. (2008). Adverse effects of chronic circadian desynchronization in animals in a "challenging" environment. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 295*(6), R2034-R2040. doi:10.1152/ajpregu.00118.2008

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMed research international, 2014*, 1-19. doi:10.1155/2014/761264

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science, 247*(4945), 975-978. doi:10.1126/science.2305266

Reinhardt, É. L., Fernandes, P. A. C. M., Markus, R. P., & Fischer, F. M. (2018). Night work effects on salivary cytokines TNF, IL-1β and IL-6. *Chronobiology international, 36*, 1-26. doi:10.1080/07420528.2018.1515771

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature, 418*(6901), 935-941. doi:10.1038/nature00965

Sand, O., Sjaastad, Ø., & Haug, E. (2014). *Menneskets fysiologi* (Vol. 2). Oslo Gyldendal Norsk Forlag AS.

Sanders, K. M., Koh, S. D., & Ward, S. M. (2006). Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. *Annu. Rev. Physiol., 68*, 307-343. doi:10.1146/annurev.physiol.68.040504.094718

Sato, S., Sakurai, T., Ogasawara, J., Shirato, K., Ishibashi, Y., Oh-ishi, S., . . . Izawa, T. (2014). Direct and indirect suppression of interleukin-6 gene expression in murine macrophages by nuclear orphan receptor REV-ERBα. *The Scientific World Journal, 2014*, 1-10. doi:10.1155/2014/685854

Scheving, L. A. (2000). Biological clocks and the digestive system. *Gastroenterology, 119*(2), 536-549. doi:10.1053/gast.2000.9305

Shehadul Islam, M., Aryasomayajula, A., & Selvaganapathy, P. R. (2017). A review on macroscale and microscale cell lysis methods. *Micromachines, 8*(3), 2-27. doi:10.3390/mi8030083

Singh, R., Singh, R., Tripathi, A., Cornelissen, G., Schwartzkopff, O., Otsuka, K., & Halberg, F. (2005). Chronomics of circulating plasma lipid peroxides and anti-oxidant enzymes and other related molecules in cirrhosis of liver: In the memory of late Shri Chetan Singh. *Biomedicine & pharmacotherapy, 59*, S229-S235. doi:10.1016/S0753-3322(05)80037-X

Sládek, M., Rybová, M., Jindráková, Z., Zemanová, Z., Polidarová, L., Mrnka, L., . . . Sumová, A. (2007). Insight into the circadian clock within rat colonic epithelial cells. *Gastroenterology, 133*(4), 1240-1249. doi:10.1053/j.gastro.2007.05.053

Solt, L. A., Kojetin, D. J., & Burris, T. P. (2011). The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis. *Future medicinal chemistry, 3*(5), 623-638. doi:10.4155/fmc.11.9

Stephan, F. K., & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Sciences, 69*(6), 1583-1586. doi:10.1073/pnas.69.6.1583

Stokes, K., Cooke, A., Chang, H., Weaver, D. R., Breault, D. T., & Karpowicz, P. (2017). The circadian clock gene BMAL1 coordinates intestinal regeneration. *Cellular and molecular gastroenterology and hepatology, 4*(1), 95-114. doi:10.1016/j.jcmgh.2017.03.011

Summa, K. C., Voigt, R. M., Forsyth, C. B., Shaikh, M., Cavanaugh, K., Tang, Y., . . . Keshavarzian, A. (2013). Disruption of the circadian clock in mice increases intestinal permeability and promotes alcohol-induced hepatic pathology and inflammation. *PloS one, 8*(6), e67102. doi:10.1371/journal.pone.0067102

Tache, Y., Martinez, V., Wang, L., & Million, M. (2004). CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity:

implications for irritable bowel syndrome. *British journal of pharmacology, 141*(8), 1321-1330. doi:10.1038/sj.bjp.0705760

Tavakkolizadeh, A., Berger, U. V., Shen, K. R., Levitsky, L. L., Zinner, M. J., Hediger, M. A., . . . Rhoads, D. B. (2001). Diurnal rhythmicity in intestinal SGLT-1 function, V max, and mRNA expression topography. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 280*(2), G209-G215. doi:10.1152/ajpgi.2001.280.2.G209

Teixeira, K. R., dos Santos, C. P., de Medeiros, L. A., Mendes, J. A., Cunha, T. M., De Angelis, K., . . . Crispim, C. A. (2019). Night workers have lower levels of antioxidant defenses and higher levels of oxidative stress damage when compared to day workers. *Scientific reports, 9*(1), 4455. doi:10.1038/s41598-019-40989-6

Torsvall, L., Akerstedt, T., Gillander, K., & Knutsson, A. (1989). Sleep on the night shift: 24-hour EEG monitoring of spontaneous sleep/wake behavior. *Psychophysiology, 26*(3), 352-358. doi:10.1111/j.1469-8986.1989.tb01934.x

Tortora, G. J., & Derrickson, B. H. (2008). *Principles of anatomy and physiology* (12 ed.). Hoboken, New Jersey: John Wiley & Sons.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nature reviews immunology, 9*(11), 799. doi:10.1038/nri2653

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology, 3*(7), research0034. 0031. doi:10.1186/gb-2002-3-7-research0034

Varga, F. (1976). Transit time changes with age in the gastrointestinal tract of the rat. *Digestion, 14*(4), 319-324. doi:10.1159/000197947

Vaze, K. M., & Sharma, V. K. (2013). On the adaptive significance of circadian clocks for their owners. *Chronobiology international, 30*(4), 413-433. doi:10.3109/07420528.2012.754457

Wang, S., Lin, Y., Yuan, X., Li, F., Guo, L., & Wu, B. (2018). REV-ERBα integrates colon clock with experimental colitis through regulation of NF-κB/NLRP3 axis. *Nature communications, 9*(1), 4246. doi:10.1038/s41467-018-06568-5

Wang, X., Ji, A., Zhu, Y., Liang, Z., Wu, J., Li, S., . . . Xie, L. (2015). A meta-analysis including dose-response relationship between night shift work and the risk of colorectal cancer. *Oncotarget, 6*(28), 25046-25060. doi:10.18632/oncotarget.4502

Welgan, P., Meshkinpour, H., & Beeler, M. (1988). Effect of anger on colon motor and myoelectric activity in irritable bowel syndrome. *Gastroenterology, 94*(5), 1150-1156. Retrieved from: https://www.gastrojournal.org/article/0016-5085(88)90006-6/abstract

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron, 14*(4), 697-706. doi:10.1016/0896-6273(95)90214-7

Wickwire, E. M., Geiger-Brown, J., Scharf, S. M., & Drake, C. L. (2017). Shift work and shift work sleep disorder: clinical and organizational perspectives. *Chest, 151*(5), 1156-1172. doi:10.1016/j.chest.2016.12.007

Williams, C. L., Villar, R. G., Peterson, J. M., & Burks, T. F. (1988). Stress-induced changes in intestinal transit in the rat: a model for irritable bowel syndrome. *Gastroenterology, 94*(3), 611-621. Retrieved from: https://www.gastrojournal.org/article/0016-5085(88)90231-4/abstract

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R.-i., Ueda, M., . . . Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science, 288*(5466), 682-685. doi:10.1126/science.288.5466.682

Yerushalmi, S., & Green, R. M. (2009). Evidence for the adaptive significance of circadian rhythms. *Ecology letters, 12*(9), 970-981. doi:10.1111/j.1461-0248.2009.01343.x

6 Appendix

6.1 Appendix A - Feces sample weights

Group	Rat ID	Weight: Glutathione peroxidase 1 + interleukin α (g)	Weight Calprotectin (g)
AW	1	0,1014	0,1122
AW	7	0,1155	0,0994
AW	10	0,102	0,1047
AW	11	0,1425	0,1406
AW	6	0,1042	0,1
AW	14	0,109	0,1228
AW	19 **	0,1249	0,1277
RW	5*	0,1027	0,1023
RW	3	0,1041	0,1101
RW	9	0,1195	0,11
RW	15*	0,1062	0,1022
RW	20	0,1026	0,1021
RW	4	0,1	0,1064
RW	8	0,1019	0,101
RW	12	0,1008	0,1039
RW	13	0,102	0,1097
RWC	3	0,1056	0,1163
RWC	6	0,1028	0,1087
RWC	11	0,1059	0,1128
RWC	13	0,1042	0,1101

RWC	5	0,1173	0,1055
RWC	9	0,1107	0,1014
RWC	1	0,1089	0,105
RWC	16	0,1047	0,1077
AWC	10	0,1072	0,1
AWC	7	0,1082	0,1113
AWC	12	0,1088	0,1106
AWC	8	0,1241	0,1091
AWC	2	0,1087	0,101
AWC	15	0,1064	0,1108
AWC	4	0,1259	0,111
AWC	14	0,1019	0,1088
RWB	18	0,1037	0,1013
RWB	19	0,107	0,1095
RWB	20	0,1174	0,121
RWB	21	0,1154	0,1094
RWB	22	0,1161	0,1005
RWB	27	0,105	0,1137
RWB	29	0,106	0,1126

6.2 Appendix B - mRNA isolation - nucleic acid concentration values

Table B1, Duodenum mucosa tissue

Test nr	Rat ID	Group	Date RNA isolation	Sample weight	Nucleic Acid Conc.	Unit
1	RW 20	RW	14.05.19	0,14276	130,9	ng/µl
2	AW 10	AW	14.05.19	0,07305	401,4	ng/µl
3	RWC 13	RWC	14.05.19	0,12183	37,3	ng/µl
4	AWC 8	AWC	14.05.19	0,15748	743,2	ng/µl
5	RW 8	RW	14.05.19	0,08082	1039,5	ng/µl
6	AW 14	AW	14.05.19	0,10832	246,9	ng/µl
7	RWC 9	RWC	14.05.19	0,20853	52,7	ng/µl
8	AWC 15	AWC	14.05.19	0,2582	359,3	ng/µl
9	RW 12	RW	15.5.19	0,09098	826,6	ng/µl
10	AW 11	AW	15.05.19	0,15986	25,5	ng/µl
11	RWC 16	RWC	15.05.19	0,1662	148,1	ng/µl
12	AWC 14	AWC	15.05.01	0,2409	14,8	ng/µl
13	RW 15	RW	15.05.19	0,0783	783,1	ng/µl
14	AW 1	AW	15.05.19	0,1013	798,6	ng/µl
15	RWC 11	RWC	15.05.19	0,1948	36	ng/µl
16	AWC 12	AWC	15.05.19	0,1413	49,6	ng/µl
17	RW 5	RW	15.05.19	0,1543	243,5	ng/µl
18	AW 6	AW	15.05.19	0,1248	1116,2	ng/µl
19	RWC 3	RWC	15.05.19	0,1114	223,5	ng/µl

20	AWC 10	AWC	15.05.19	0,2162	73	ng/µl
21	RW 3	RW	15.05.19	0,15912	141,4	ng/µl
22	AW 19	AW	15.05.19	0,12682	847	ng/µl
23	RWC 5	RWC	15.05.19	0,20371	205	ng/µl
24	AWC 2	AWC	15.05.19	0,15778	760,2	ng/µl
25	RW 9	RW	15.05.19	0,0594	385,4	ng/µl
26	AW 7	AW	15.05.19	0,1246	477,9	ng/µl
27	RWC 1	RWC	15.05.19	0,1818	554,9	ng/µl
28	AWC 4	AWC	15.05.19	0,2372	393	ng/µl
29	RW 13	RW	15.05.19	0,0805	459,2	ng/µl
30	RWC 6	RWC	15.05.19	0,1942	850,1	ng/µl
31	AWC 7	AWC	15.05.19	0,1906	466,4	ng/µl
32	RW 4	RW	15.05.19	0,1022	1280,1	ng/µl

Table B2, Colon mucosa tissue

Test nr	Rat ID	Group	Date RNA isolation	Sample weight	Nucleic Acid Conc.	Unit
1	RW 15	RW	16.05.19	0,12412	356,5	ng/µl
2	AW 11	AW	16.05.19	0,13566	51,4	ng/µl
3	RWC 16	RWC	16.05.19	0,26332	76,6	ng/µl
4	AWC 14	AWC	16.05.19	0,23544	190,8	ng/µl
5	RW 8	RW	16.05.19	0,1769	169,1	ng/µl
6	AW 7	AW	16.05.19	0,15105	104,2	ng/µl

7	RWC 13	RWC	16.05.19	0,23227	107,1	ng/µl
8	AWC 8	AWC	16.05.19	0,1979	141,6	ng/µl
9	RW 20	RW	16.05.19	0,13912	543,3	ng/µl
10	AW 6	AW	16.05.19	0,17971	194,9	ng/µl
11	RWC 6	RWC	16.05.19	0,17491	120,9	ng/µl
12	AWC 7	AWC	16.05.19	0,16129	211,2	ng/µl
13	RW 4	RW	16.05.19	0,184	135,7	ng/µl
14	AW 10	AW	16.05.19	0,1219	154,6	ng/µl
15	RWC 5	RWC	16.05.19	0,18094	158,7	ng/µl
16	AWC 2	AWC	16.05.19	0,25197	89,7	ng/µl
17	RW 9	RW	16.05.19	0,18563	355,3	ng/µl
18	AW 19	AW	16.05.19	0,13418	84,1	ng/µl
19	RWC 11	RWC	16.05.19	0,22649	64,4	ng/µl
20	AWC 12	AWC	16.05.19	0,24654	51,4	ng/µl
21	RW 5	RW	16.05.19	0,12245	470,3	ng/µl
22	AW 1	AW	16.05.19	0,13663	197,5	ng/µl
23	RWC 1	RWC	16.05.19	0,1993	71,6	ng/µl
24	AWC 4	AWC	16.05.19	0,20905	185,1	ng/µl
25	RW 13	RW	16.05.19	0,18296	141,9	ng/µl
26	AW 14	AW	16.05.19	0,18449	44,5	ng/µl
27	RWC 3	RW	16.05.19	0,17886	286,9	ng/µl
28	AWC 10	AWC	16.05.19	0,12614	134,1	ng/µl
29	RW 3	RW	16.05.19	0,17999	143,5	ng/µl

30	RWC 9	RWC	16.05.19	0,1639	160,1	ng/µl
31	AWC 15	AWC	16.05.19	0,26446	59,9	ng/µl
32	RW 12	RW	16.05.19	0,19024	189,8	ng/µl